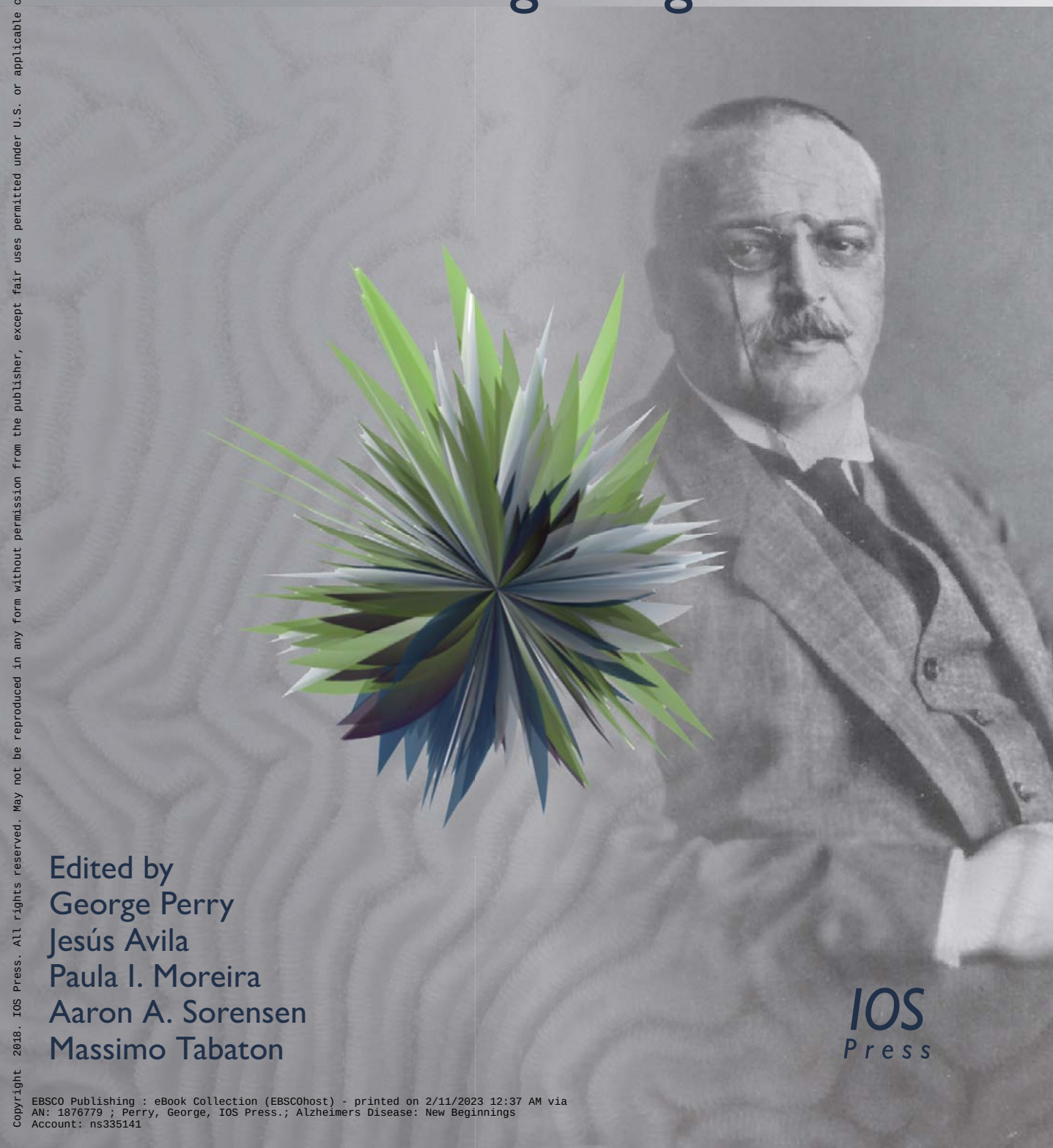
ADVANCES IN ALZHEIMER'S DISEASE 6

Alzheimer's Disease: New Beginnings



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IOS Press

ADVANCES IN ALZHEIMER'S DISEASE 6

Series editors: George Perry Ph.D and J. Wesson Ashford, MD, PhD

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Alzheimer's Disease: New Beginnings focuses on the future promise for therapeutic breakthroughs in light of notable clinical trial failures. The volume editors used a combination of scientometric evaluations to determine the most promising new approaches as well as soliciting insights from leaders in each of the major areas of Alzheimer's disease research. By combining these two approaches, they recruited authors from the entire outlook spectrum, from those who feel an elusive breakthrough might still be a few, well-placed tweaks away to those who feel that they are launching entirely new investigative paradigms. These scholars present an open-eyed path forward. Now is the most exciting period in this field as old dogmas make way for new insights, from new approaches to clinical trials, improved biomarker-based diagnostics, population-based studies, prevention, metabolism, to further refinement of the role of inflammation, genetics, tau, and amyloid-β.

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ALZHEIMER’S DISEASE: NEW BEGINNINGS

Advances in Alzheimer's Disease

Advances in Alzheimer's Disease brings together the latest insights in Alzheimer's disease research in specific areas in which major advances have been made. This book series assembles and builds on work recently published in the *Journal of Alzheimer's Disease* (JAD) and also includes further contributions to ensure comprehensive coverage of the topic. The emphasis is on the development of novel approaches to understanding and treating Alzheimer's and related diseases.

Series Editors:
George Perry, Ph.D. and J. Wesson Ashford, M.D., Ph.D.

Volume 6

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Preface

Alzheimer's Disease: New Beginnings focuses on the future promise for therapeutic breakthroughs in light of notable clinical trial failures. We used a combination of scientometric evaluations to determine the most promising new approaches as well as soliciting insights from leaders in each of the major areas of Alzheimer's disease research. By combining these two approaches, we recruited authors from the entire outlook spectrum of those who feel an elusive breakthrough might still be a few, well-placed tweaks away to those who feel that they are launching entirely new investigative paradigms. These scholars present an open-eyed path forward. Now is the most exciting period of a generation in our field as old dogmas make way for new insight, whether it be new approaches to clinical trials, improved biomarker-based diagnostics, population-based studies, prevention, metabolism, or further refinement of the role of inflammation, genetics, tau, and amyloid-β.

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Clinical Trials for Disease-Modifying Therapies in Alzheimer's Disease: A Primer, Lessons Learned, and a Blueprint for the Future

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Abstract. Alzheimer's disease (AD) has no currently approved disease-modifying therapies (DMTs), and treatments to prevent, delay the onset, or slow the progression are urgently needed. A delay of 5 years if available by 2025 would decrease the total number of patients with AD by 50% in 2050. To meet the definition of DMT, an agent must produce an enduring change in the course of AD; clinical trials of DMTs have the goal of demonstrating this effect. AD drug discovery entails target identification followed by high throughput screening and lead optimization of drug-like compounds. Once an optimized agent is available and has been assessed for efficacy and toxicity in animals, it progresses through Phase I testing with healthy volunteers, Phase II learning trials to establish proof-of-mechanism and dose, and Phase III confirmatory trials to demonstrate efficacy and safety in larger populations. Phase III is followed by Food and Drug Administration review and, if appropriate, market access. Trial populations include cognitively normal at-risk participants in prevention trials, mildly impaired participants with biomarker evidence of AD in prodromal AD trials, and subjects with cognitive and functional impairment in AD dementia trials. Biomarkers are critical in trials of DMTs, assisting in participant characterization and diagnosis, target engagement and proof-of-pharmacology, demonstration of disease-modification, and monitoring side effects. Clinical trial designs include randomized, parallel group; delayed start; staggered withdrawal; and adaptive. Lessons learned from completed trials inform future trials and increase the likelihood of success.

Keywords: Alzheimer's disease, biomarkers, clinical trials, disease modifying therapies, proof-of-concept, target engagement

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that produces gradual decline in cognition and function [1, 2]. The most common form, late onset AD, becomes symptomatic in late life but biomarker studies show that the amyloid protein considered the major risk factor for the disease begins to accumulate in the brain up to 20 years before symptoms begin [3].

The total number of individuals with AD will double every 20 years [4]. The annual cost of AD currently exceeds $230 billion and the total annual cost will exceed $1 trillion by 2050 if means of preventing, delaying, slowing the progression, or improving the symptoms are not found [5].

There is a high rate of negative clinical trials in AD drug development programs; 99% of drugs tested

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between 2002 and 2014 showed no drug-placebo difference and only one drug was approved by the US Food and Drug Administration (FDA) during that period [6]. Drugs in the AD pipeline include agents intended to intervene in the basic biology of AD and modify disease progression, symptomatic cognitive enhancers, and drugs to treat neuropsychiatric symptoms [7, 8].

The greatest need in AD drug development is for disease-modifying therapies (DMTs) that will delay or slow the clinical course of AD by intervening in the processes leading to cell death [9]. Approximately two-thirds of the current AD drug development pipeline involves DMTs—either immunotherapies or small molecule agents administered orally [7, 8]. In this paper, we describe the methods for AD clinical trials of DMTs, review past failures to identify lessons for AD drug development, and look ahead to new approaches to improving AD drug development and optimizing success in bringing new treatments to patients with AD or those at high risk for the disorder.

OVERVIEW

Figure 1 shows the overview of an AD treatment discovery and development program beginning with identification of a target and proceeding through preclinical (sometimes called non-clinical) characterization; to Phase I, Phase II, and Phase III clinical trials; and to regulatory review and patient access through marketing. On average, development for an AD treatment requires 13 years and is expected to cost $5.6 billion U.S. dollars. Preclinical evaluation requires approximately 2 years, Phase I averages 2.8 months, Phase II requires 27.7 months, Phase III is typically 50.9 months, and FDA review requires 18 months [10]. These figures are for AD drugs of all types and likely under-estimate the time taken to develop an AD DMT.

The biography of new agents can be divided into *discovery* phases extending from the first characterization of the compound to the final optimization of the lead candidate and *development* extending from preclinical/animal testing to Phase I First-in-Human (FIH) studies through Food and Drug Administration (FDA) review and to Phase IV for those agents undergoing post-approval assessment.

DRUG DISCOVERY

Target identification and drug discovery

A DMT must intervene in the basic biology of AD leading to cell death [9]. Common targets in AD are processes of production, oligomerization, or clearance of the amyloid-β protein (Aβ); the development of neurofibrillary tangles from the tau protein; processes associated with cellular metabolism; neuroinflammation; oxidative injury to membranes; or cell maintenance and regeneration strategies such as

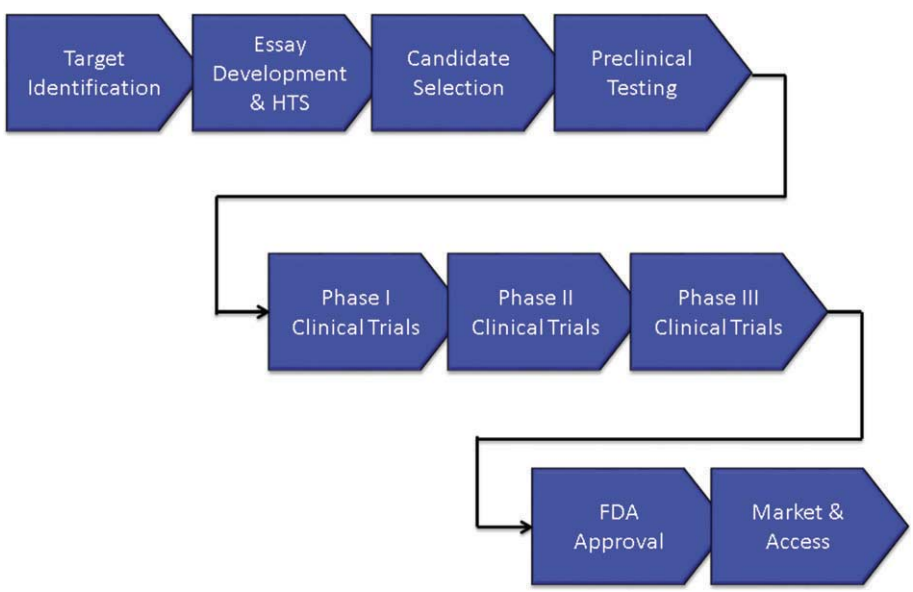


Fig. 1. Overview of the drug development process.

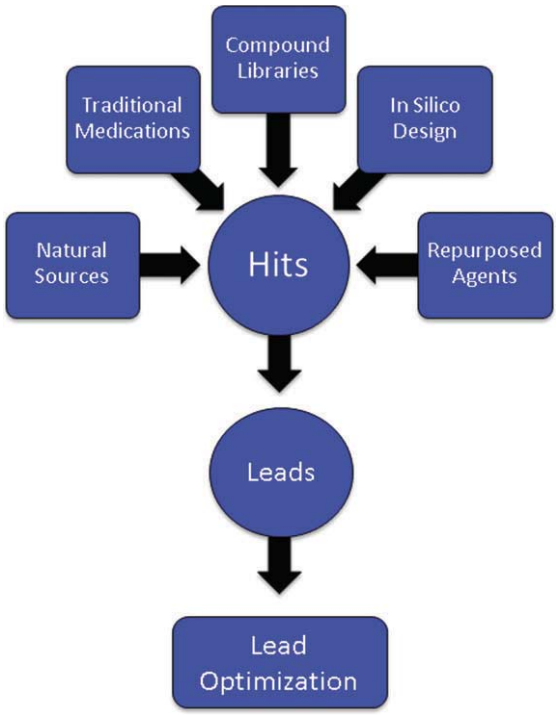


Fig. 2. Origin of compounds that are assayed through high throughput screening to produce "hits" that are then subject to medicinal chemistry refinement to produce leads and optimized leads.

stem cells or growth factors. Targets must be "druggable" to provide the basis for a drug discovery and development programs; druggable targets have properties that can be modulated by small molecules (e.g., drugs) or antibodies [11].

After a target has been identified, an assay is developed for the proposed mechanism of action (MOA) such as inhibition of the β-secretase enzyme necessary for Aβ production, modulation of the γ-secretase enzyme also critical to Aβ generation, inhibition of oligomerization of Aβ into its most toxic form, phosphorylation of the tau protein required for the formation of neurofibrillary tangles, activation of microglia in the inflammatory process, or manipulation of cell survival through growth factors. Large numbers of compounds ("libraries") are screened for "hits" that have the desired effects in the assay (Fig. 2). Libraries are constructed from pharmacophores with multiple molecular forms, traditional medications (e.g., Traditional Chinese Medicines), natural sources and biodiversity, repurposed agents that may have AD-related effects, and compounds designed by computer where structure-activity relationships can be modeled *in silico* [12]. Several hundred thousand compounds may be screened to identify a sufficient number of hits to provide a foundation for further development. The hits are reviewed by medicinal chemists for "drug-likeness" including features that predict good absorption and membrane penetration [13, 14]. Agents with promising characteristics are optimized for molecular features that enhance the likelihood of being successful as a drug for human therapy—potency, half-life, predictable toxicity, blood-brain barrier (BBB) penetration, etc. Once a lead compound and several backups are identified, testing in animals can begin [15].

An alternative to high-throughput screening with biological assays is high content analysis (HCA), conducted in intact cells using automated microscopy and image analysis. HCA can be used to screen for effects on protein aggregation, synaptic integrity, neuron and synapse number, and apoptosis as well as other cellular processes relevant to AD treatment [16]. HCA may more closely reflect the neurological environment in which drugs must act when administered in the human setting.

Preclinical assessment

Assessment of the lead candidate in animals establishes the pharmacokinetic characteristics, toxicity, and efficacy of the molecule in the test species. Testing involves both short-term and long-term treatment in a wide range of doses to establish the absorption, distribution, metabolism, excretion (ADME), and toxicity of the potential treatment [17]. Testing is required in two species, usually mice and rats. Dogs have a high sensitivity to cardiac effects of drugs and are the usual assay species for cardiac toxicity [18]. Special attention is paid to liver and bone marrow toxicity; laboratory and necropsy studies are performed to thoroughly assess any off-target adverse effects in the animals. In addition, panels of enzymes, ion channels, and other biological mechanisms are used to search for unanticipated off-target effects of the candidate therapy [19]. If no unusual toxicity is identified, the highest drug dose level at which no adverse events (NOAEL) are seen is determined and becomes the basis for dose calculations for the maximum recommended safe starting dose (MRSD) for FIH studies [20].

Development of monoclonal antibodies (mAb) differs from that of the approach to developing small molecules. Monoclonal antibodies are manufactured to interact with a specific epitope of a target such as a portion of the Aβ molecule to limit is oligomerization into a more toxic form, facilitate its removal

by brain microglia, or bind with peripheral Aβ to form a "peripheral sink" to remove AD from the brain [21, 22]. Monoclonal antibodies have fewer risks for off-target effects since they are exquisitely targeted to specific molecular sites.

Animal species are also used to explore the efficacy of candidate therapies. Throughout the drug development process, every effort is made to minimize the use of animals and to develop alternatives to animal observations in the assessment of both the toxicity and efficacy of candidate therapies. Although success in animal models has not yet predicted success of a DMT in humans, the failure to see the desired effect in an animal model system of AD biology would constitute a reason not to advance the molecular candidate to human testing [23]. The most commonly used animal model systems are transgenic (tg) mice that have one or more human genes known to cause familial AD in their genome. The amyloid precursor protein/presenilin 1 double tg is a widely used test animal. These genetically modified mice begin to deposit brain amyloid by 6 months of age and by 9 months of age show mild cognitive impairment. Anti-amyloid approaches can be tested in this model. Triple tg and 5x tg as well as many types of gene knock-in (KI) and knock-out (KO) species have been developed. The model animals exhibit specific aspects of the AD pathology observed in humans. Tg animals develop brain amyloidosis with plaques similar to those of humans but typically have little tau formation, inflammation, or cell death characteristic of human AD. They provide a means of assessing the anti-amyloid effect of the agent but not its likely success in the complex multifactorial AD process observed in humans [24].

Human-derived induced pluripotent stem (iPS) cells are increasingly used to screen drugs and to move the early screening process toward a more human biological context with the hope of having greater predictability for human response. The stem cells may be derived from fibroblasts of patients with autosomal dominant AD and the induced stem cells undergo directed transformation to neurons which bear the genetic abnormality and can be the substrate for drug efficacy assessment, or skin cells from unaffected donors can be transformed into iPS cells and then into neurons and an amyloid-related mutation is introduced to create a platform for treatment assessment [25, 26]. The cells are grown in gels allowing 3-dimensional growth and spontaneously form organoid structures with brain-like features. The iPS cell platforms show both amyloid and tau protein accumulation further recapitulating the human disease and creating a more ecologically valid system for drug efficacy assessment [25].

If the candidate agent has acceptable ADME and toxicity characteristics and shows desirable activity in the model used to assess efficacy, it will be advanced to human testing.

CLINICAL TRIALS

Introduction

The development phase—and to a lesser extent the discovery phase—of drug creation is guided by a Target Product Profile (TPP) [27]. The TPP defines the desirable features of a drug and its use including the primary indication, patient population, treatment duration, delivery mode, dosage, regimen, tolerability, risk/side effects, tolerability, and differentiating features in a competitive landscape. A minimally acceptable profile and an ideal profile are identified. Failure to achieve the minimally acceptable profile may lead to discontinuation of the development program. Using the TPP, the indication and proposed package insert are constructed and the development program is designed in reverse to insure that all the features of the TPP are fully defined for the compound in the course of development.

Clinical trials must be reported in a specific format called the Consolidated Standards of Reporting Trials (CONSORT) when they are submitted to journals [28]. The International Committee of Medical Journal Editors has subscribed to these requirements to achieve standardized reporting of clinical trials. Table 1 provides the CONSORT checklist of elements to be included in any report of a clinical trial. Anticipation of the features to be reported allows the checklist to function as a useful guide to planning a clinical trial.

The National Institutes of Health (NIH) has developed a template that can be used to plan a clinical trial including all elements necessary to meet Good Clinical Practice (GCP) guidelines and CONSORT requirements (http://osp.od.nih.gov/sites/default/files/Protocol_Template_05Feb2016_508.pdf). This extensive template serves as a precise guide to clinical trial planning and presentation for Institutional Review Board, funder, and FDA review.

Phase I

Phase I involves the FIH exposure of the drug. In small molecule development programs, the persons

Table 1
CONSORT checklist [28]

Section / Topic	Checklist Item
Introduction	
Background and objectives	Scientific background and explanation of rationale; specific objectives or hypotheses
Methods	
Trial design	Description of trial design (such as parallel, factorial) including allocation ratio; important changes to methods after trial commencement (such as eligibility criteria), with reasons
Participants	Eligibility criteria for participants; settings and locations where the data were collected
Interventions	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered
Outcomes	Completely defined pre-specified primary and secondary outcome measures, including how and when they were accessed; any changes to trial outcomes after the trial commenced, with reasons
Sample size	How sample size was determined; When applicable, explanation of any interim analyses and stopping guidelines
Random sequence generation	Method used to generate the random allocation sequence; type of randomization; details of any restriction (such as blocking and block size)
Allocation concealment mechanism	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers)
Randomization implementation	Who generated the random allocation sequence, who enrolled the participants, and who assigned participants to interviews
Blinding	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and; if relevant, description of the similarity of interventions
Statistical methods	Statistical methods used to compare groups for primary and secondary outcomes
Results	
Participant flow diagram	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analyzed for the primary outcome; for each group, losses and exclusions after randomization, together with reasons
Recruitment	Dates defining the periods of recruitment and follow-up
Baseline data	A table showing baseline demographic and clinical characteristics for each group
Numbers analyzed	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned group
Outcomes and estimation	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval); for binary outcomes, presentation of both absolute and relative effect sizes
Ancillary analyses	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory
Harms	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms (28)
Discussion	
Limitations	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses
Generalizability	Generalizability (external validity, applicability) of the trial findings
Interpretation	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence
Other Information	
Registration	Registration number and name of trial registry
Funding	Sources of funding and other support (such as supply of drugs); role of funders

participating in the Phase I trial are normal healthy volunteers [29]. If a mAb or vaccine is being developed, the FIH testing is usually done with patients with AD. Immunotherapies can permanently alter the immune system—this is more likely with a vaccine than a mAb—and the unknown consequences of this cannot be risked in young healthy individuals.

Single ascending dose (SAD) studies where cohorts of individuals are exposed to a single dose of progressively higher doses of the agent are followed by multiple ascending dose (MAD) studies where cohorts are treated for 14–28 days with progressively higher doses of the agent [30]. A cohort is typically 8–12 individuals randomized in a 4:1 ratio of active agent to placebo. SAD studies and some portions of MAD studies are conducted in specially designed Phase I in-patient units. Serial blood samples as well as urine and stool samples are collected to determine ADME characteristics in humans. Patient reports, physical examination, electrocardiography, and blood tests are collected to determine the safety and tolerability of each dose.

Ideally, a maximum tolerated dose (MTD) is determined at this stage of drug development. There are several ways of determining the lowest dose to be tested in Phase I; typically a dose representing 1/10 of the NOAEL observed in the most sensitive animal species is the beginning dose and the dose is doubled in each successive cohort [20]. The MTD informs future studies since it represents the upper limit of dosing. Low, medium, and high doses are typically advanced to Phase II. Failure to establish an MTD in Phase I can lead to future challenges in the development process; if later trials are negative, it may be difficult to know whether the agent is ineffective or was not given in a sufficient dose.

Assessing CSF drug levels in Phase I can provide important insights about a candidate compound's ability to penetrate the human BBB and exert CNS effects. Treatments should not exit Phase I without evidence of BBB penetration and an understanding of plasma/CSF ratios. Consisting of tight junctions joining the endothelial cells of the central nervous system, the BBB creates a physical barrier that severely restricts the size and ionic properties of molecules permitted to cross into the brain [31]. Augmenting the physical barrier is a complex network of enzymes and transport proteins, such as P-glycoprotein (P-gp), breast cancer resistance protein, and multidrug resistance protein that metabolize and expel molecules that are able to pass through the physical barrier. The BBB thus represents a significant obstacle for agents intended to reach targets deep within the brain parenchyma and failure to penetrate the BBB has contributed to failed development programs [32].

The challenges of BBB penetration require confirmation of drug delivery into the brain in early phase testing. Lumbar CSF measures provide an approximation of the brain exposure in humans. Levels of unbound, pharmacologically active drug in CSF can be drawn during continuous intravenous infusion or at fixed time points after systemic delivery [33]. Differences in the human and rodent BBB, particularly the robustness of the P-gp system, leads to differences in human CSF levels and makes extrapolations between human and animal data problematic [34]. Observations made from CSF in the healthy state must later be confirmed in the disease state as differences in cerebral blood flow, activity of efflux transport proteins, and BBB permeability with disease may fundamentally alter drug delivery [35].

Phase II

Drugs that appear safe and have acceptable ADME and safety profiles when tested in normal human volunteers are advanced to Phase II to be tested in the population of interest, AD. Repurposed agents that have been gone through Phase I while being developed for another indication (e.g., hypertension, cancer, Parkinson's disease, diabetes, etc.) may enter directly into Phase II or occasionally directly into Phase III [36, 37].

Phase II generally encompasses Phase IIa proof-of-concept (POC) trials and Phase IIb dose-finding studies. The goal of Phase II is to gain confidence in the treatment and provide information for Phase III trials. Phase II involves patients with AD dementia or prodromal AD [38]. A conundrum has evolved for Phase II trials of AD DMTs. The decision to advance an agent to Phase II could be based on a Phase IIa study with a biomarker outcome, using the biomarker to decide if there is a sufficient likelihood of clinical success. The challenge with this approach is that there is no AD biomarker that has gained surrogate status and none is known to predict a clinical outcome. Alternately, one can require clinical POC with benefit on a traditional clinical measure such as the AD Assessment Scale – cognitive portion (ADAS-cog) [39] or Clinical Dementia Rating – Sum of Boxes (CDR-sb) [40]. To show clinical benefit typically requires a large long trial equivalent to a Phase III trial [41]. Thus, some development programs move from Phase I directly to Phase III. This often results in a Phase III program that is advancing an agent with limited information regarding safety, tolerability, biomarker effects, or dosing. This strategy may contribute to the high failure rate of AD drug development and the absence of any successful DMTs [6].

Increasingly, biomarkers are used in Phase II to support decision making for development programs (Fig. 3). Biomarkers are used to confirm the diagnosis of AD. The clinical diagnosis of AD dementia is not confirmed by amyloid or CSF amyloid and tau measures in approximately 25% of patients diagnosed clinically with AD [42], indicating that they do not have the pathobiology of AD. Approximately 50% of mild cognitive impairment patients have abnormal amyloid measures and constitute a prodromal AD population—50% do not have early AD [43]. AD trials must have individuals with AD to draw accurate conclusions about efficacy of AD-directed therapies. Figure 4 shows normal and AD-type amyloid positron

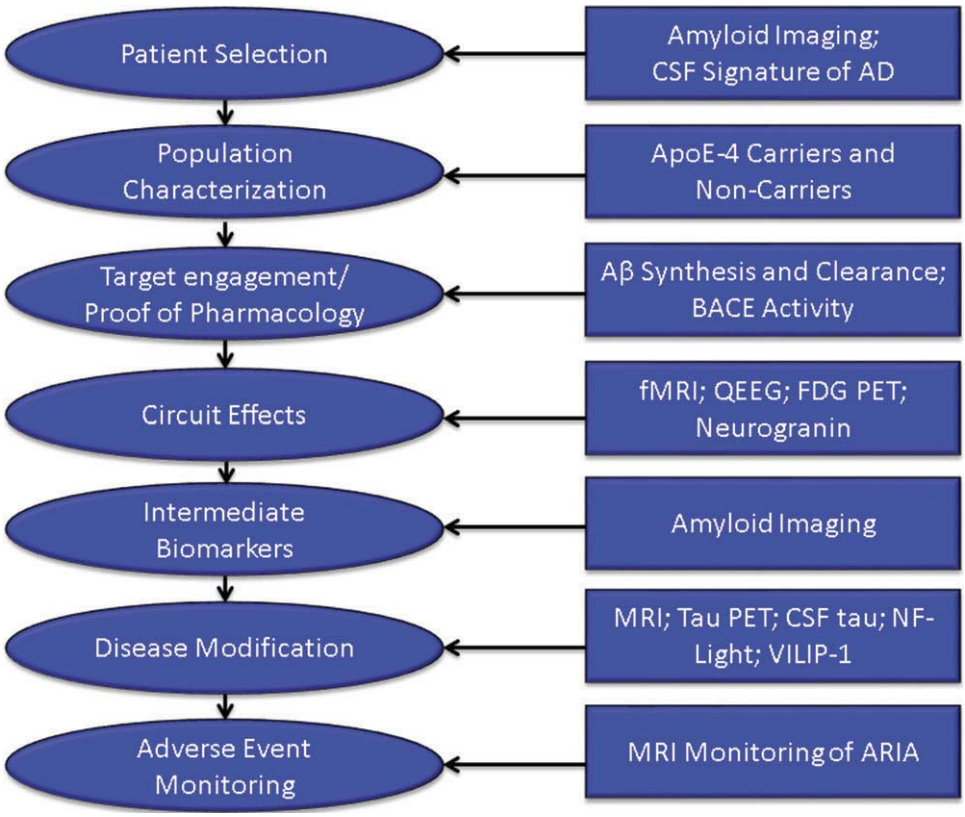


Fig. 3. Roles of biomarkers in Phase II of drug development (BACE inhibition is included as an example of one type of target engagement biomarker; each drug mechanism will have a corresponding target engagement/proof of pharmacology biomarker), CSF, cerebrospinal fluid; AD, Alzheimer's disease; fMRI, functional magnetic resonance imaging; QEEG, quantitative electroencephalography; FDG PET, fluorodeoxyglucose positron emission tomography; NF-light, neurofilament light chain protein; ARIA, amyloid-related imaging abnormalities.

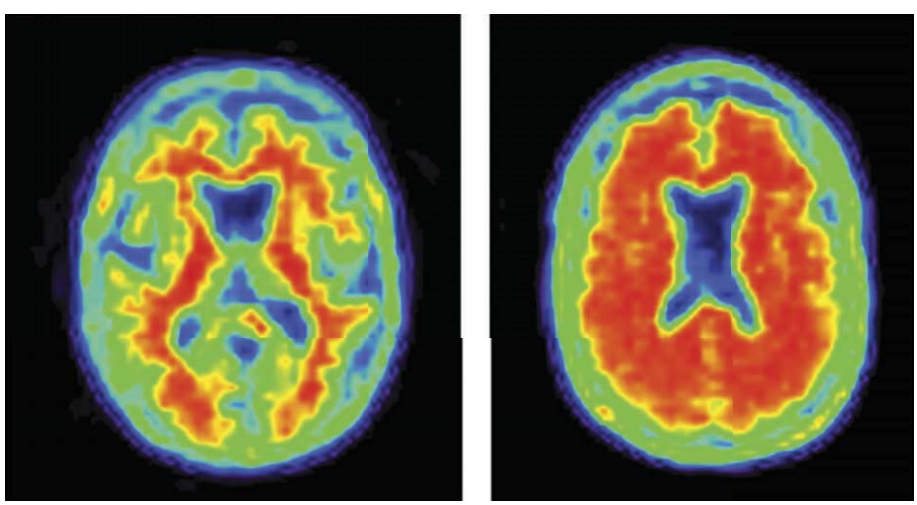


Fig. 4. Negative (normal) and positive (abnormal; consistent with AD) amyloid PET images.

emission tomography (PET) used to support the diagnosis of AD.

Populations in AD trials are typically divided by apolipoprotein E (ApoE) genotype into ApoE4 allele carriers and noncarriers. Allele status may affect efficacy and side effects and often influences dosing in mAb trials [42, 44, 45]. Recruitment may not be stratified by genotype but the statistical analysis plans will contrast carriers and noncarriers for efficacy and toxicity.

Target engagement biomarkers are critical to demonstrating that the drug is having the desired clinical effect on the near-term target. Without target engagement, the disease-modifying properties of the drug cannot be assessed. For example, if a beta-site cleavage enzyme (BACE) inhibitor is not producing BACE inhibition or is not affecting amyloid synthesis, then the hypothesis that BACE inhibition will produce disease-modification cannot be assessed. Amyloid deposition is an intermediate biomarker of drug efficacy. It may not be immediately related to cell death but appears necessary to establish an environment in which cell death occurs. Effects on amyloid deposition can serve as an intermediate biomarker of anti-plaque effects of anti-amyloid drug activity. In the PRIME study of aducanumab, for example, reduced brain amyloid was demonstrated after 6 months of therapy and was more marked after 12 months of treatment [43].

Cognition is mediated by integrated cerebral circuits and preservation of circuit integrity is a precondition for a beneficial cognitive impact of therapy. Circuit function can be assessed by fMRI or quantitative electroencephalography [46, 47]. Fluorodeoxyglucose PET reflects synaptic integrity and is a measure of circuit synaptic function [48]. Neurogranin is a measure of synaptic integrity that may represent a fluid biomarker of circuit preservation. These circuit measures can assess the circuit level impact of therapy and may better predict the cognitive outcome [49].

Biomarkers suggesting that an agent has produced disease modification are those that are closely correlated with processes leading to cell death. A drug-placebo difference in these biomarkers in favor of less degeneration and more neuroprotection by the active agent indicates that the drug is a DMT [9].

Biomarkers currently considered as indicative of disease-modification in AD include volumetric MRI as well as measures of tau protein aggregation (tau PET, CSF tau), neurofilament light chain protein, and VILIP-1 [50–54].

Finally, MRI is used to monitor amyloid-related imaging abnormalities (ARIA) occurring as a side effect in patients treated with some anti-amyloid mAbs [44]. Other biomarkers commonly used to monitor adverse events of medications include liver functions, hematologic measures, and electrocardiography.

At the end of Phase II, the ADME, safety, tolerability, and target engagement of the test agent should be known. Dosing should be narrowed to one or two doses before proceeding to Phase III. Understanding these aspects of the candidate therapy at the end of Phase II builds confidence in the therapeutic approach and makes it more likely that the agent will succeed in Phase III.

Phase III

Phase II and Phase III are often conceived as "learn" and "confirm" trials [55]. The learnings of Phase II are tested in Phase III and, if benefits are confirmed, the agent will be submitted to the FDA for review. Phase III trials for DMTs are 12 to 24 months in duration and typically involve 600–1000 patients per arm of the study (each dose and the placebo comprise 1 arm each). The reasons for failure of drugs to advance from Phase III to regulatory review include lack of efficacy (50%), unacceptable toxicity (14%), and commercial, strategic, and operational issues (31%) [56]. These figures are for all classes of agents (not limited to AD-directed drugs); they emphasize the importance of accruing efficacy data in Phase II. Drugs that have genetic connections to the neurobiology of the disease and that have biomarkers to inform drug development decisions are more likely to advance from one phase to the next than drugs that lack this information [57].

As noted above, biomarkers are used in Phase III to diagnose participants, support disease-modifying activity, and monitor amyloid-related imaging abnormalities in mAb studies.

Phase IV and post-marketing studies

Phase IV studies occur after the drug has been approved by the FDA or other regulatory agency and is available on the market. Phase IV studies may be used to extend treatment to a new indication, for example, the assessment and eventual approval of rivastigmine for the treatment of Parkinson's disease dementia after its approval for mild-moderate AD dementia [58]. Phase IV trials can also be used to

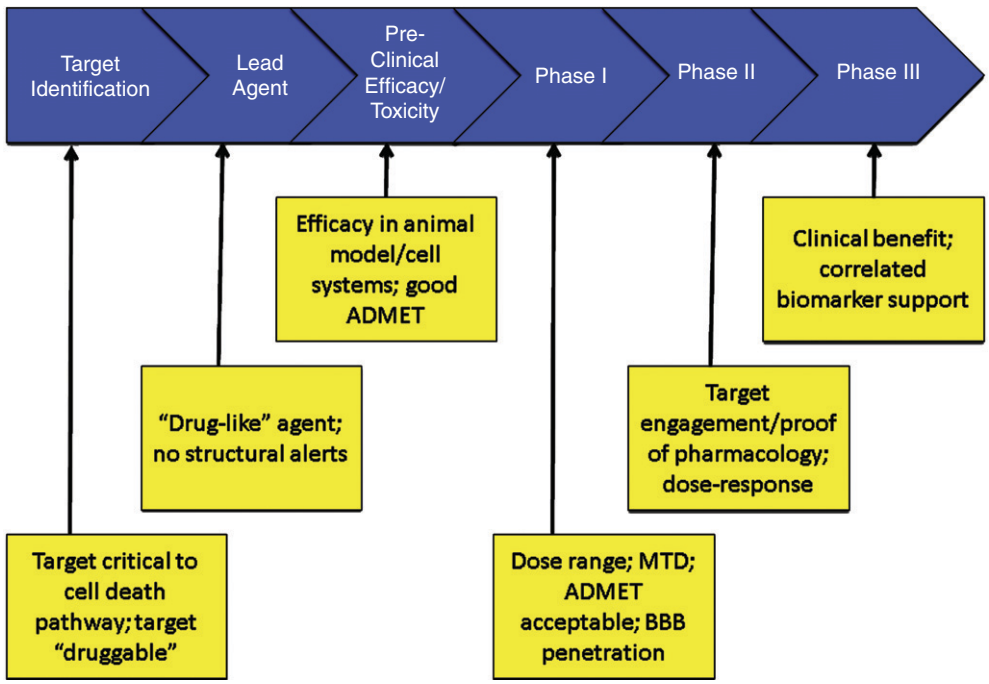


Fig. 5. Critical data to be accrued in each stage of drug discovery and development (ADMET – absorption, distribution, metabolism, excretion, toxicity; BBB – blood brain barrier; MTD – maximum tolerated dose).

extend an indication within the same disease such as the extension of donepezil and rivastigmine into severe AD after approval for mild-moderate AD [59, 60]. The FDA may also require demonstration of efficacy with Phase IV studies after approval of an agent on the basis of a change in a biomarker that is considered reasonably likely to predict a clinical benefit. This type of conditional approval is a consideration in prevention treatments where trial outcomes will emphasize biomarkers in populations without clinical symptoms.

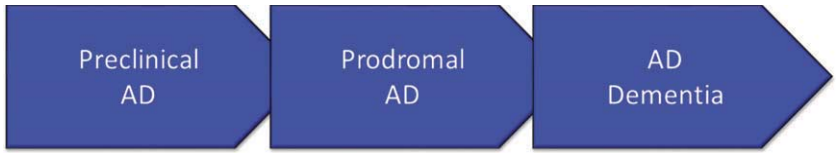
If there are safety concerns, the FDA may require the sponsor to construct a Risk Evaluation and Management Strategy (REMS) to be monitor the safety of an agent once it is marketed [61]. Figure 5 summarizes the critical data to be accrued at each stage of drug development that should be known before proceeding to the next stage.

TRIALS OF DISEASE-MODIFYING THERAPIES IN ALZHEIMER'S DISEASE

Defining disease modification

A DMT is defined as an intervention that produces an enduring change in the clinical progression of AD by interfering in the underlying pathophysiological mechanisms of the disease process that lead to neuronal death [9]. DMT efficacy is demonstrated through clinical trial designs and biomarkers. Evidence of disease modification in the drug development process is based on clinical trial designs such as staggered start and delayed withdrawal or with parallel designs incorporating combined clinical outcomes and correlated biomarker evidence of an effect on the underlying pathophysiological processes of the disease. Most development programs rely on biomarkers to provide support for DM rather than using clinical trial design strategies. The biological change associated with disease modification (DM) is neuroprotection, and biomarker support for DM depends on demonstration of neuronal preservation. DM and neuronal preservation cannot be observed directly and must be inferred from biomarker evidence. To support DM, the biomarker must be indicative of a change in the processes leading to the loss of neurons. Biomarkers commonly used in clinical trials of DMT are discussed above (Fig. 3).

DM is not equivalent to "cure" or to prevention of decline; DM refers to a permanent change in disease trajectory that will delay the onset of symptoms or slow progression in symptomatic patients.



Cognitively Normal	Episodic Memory Impairment	Dementia
Functionally Normal	Functionally Normal	Functionally Impaired
+ Amyloid Imaging; AD CSF Signature	+ Amyloid Imaging; AD CSF Signature	+ Amyloid Imaging; AD CSF Signature
MRI Normal	MRI Atrophy	MRI Marked Atrophy
Prevent/Delay Cognitive Decline	Prevent Progression to AD Dementia	Slow Progression of AD Dementia

Fig. 6. Phases of Alzheimer's disease (AD) as defined by cognitive, functional, and biomarker observations. Trial goals for each phase are noted.

A delay of 5 years would equate to decreasing the total number of affected individuals by 50%. If the treatment is available by 2025 the annual savings to the US economy by 2050 is projected to be $369 billion [62].

Populations

Phases of AD are recognized; these are not distinct stages but represent a seamless progression from a high risk state in which amyloid is present in the brain in the form of neuritic plaques, to prodromal AD with episodic memory impairment (in the typical presentation of AD) and biomarker evidence of AD, to AD dementia with cognitive and functional impairment characterized as mild, moderate or severe [38] (Fig. 6). Although these phases represent progression along a seamless spectrum of severity, they are artificially divided for purposes of clinical trials. Tools and outcomes appropriate for one phase of disease (e.g., preclinical) are not the same as those one would choose for later phases (e.g., mild-moderate AD). Table 2 provides examples of cognitive and functional measures used as outcome measures for different phases of AD [39, 40, 63–70].

Clinical outcomes in AD dementia trials are well established and have been used to demonstrate efficacy of cholinesterase inhibitors and memantine. Cognitive measures for mild-moderate AD dementia include the ADAS-cog [39] and the Neuropsychological Test Battery [66]. Common secondary measures include the CDR-sb [40], Clinical Global Impression of Change (CGIC), and the Neuropsychiatric Inventory [71]. Dual outcomes are required in AD dementia trials and include a cognitive measure with a functional or global outcome.

Prodromal trials commonly use a composite endpoint comprised of cognitive and functional elements or of cognitive elements derived from several scales. Composite endpoints include the CDR-sb [40], the AD Composite Scale (ADCOMS) [69], and the integrated AD Rating Scale (iADRS) [70]. The FDA has indicated that demonstration of both cognitive and functional benefit is necessary for drug approval in the prodromal phase of AD; a drug-placebo difference on a composite scale should not depend entirely on differences in cognition [72]. Some trials of DMTs include both patients with prodromal AD and those with mild AD dementia; the differences in these populations is arbitrary, and the groups can be usefully combined to facilitate recruitment of a broader population and show benefit in patients who have more than minimal impairment.

Prevention trials include primary prevention studies involving participants with no cognitive symptoms and no state biomarker changes of AD or secondary prevention studies including participants who have no cognitive symptoms but in whom

Table 2
Outcome tools used for the progressive phases of Alzheimer's disease [39, 40, 63–70]

Feature	Preclinical AD	Prodromal AD	AD Dementia
Cognition	Preclinical Alzheimer Cognitive Composite (PACC); Alzheimer Prevention Initiative Cognitive Composite (APCC) Test	Clinical Dementia Rating- Sum of Boxes (CDR-sb); AD Composite Score (ADCOMS); Integrated AD Rating Scale (iADRS)	Alzheimer's Disease Assessment Scale – Cognitive Subscale (ADAS-cog); Severe Impairment Battery (SIB); Neuropsychological Test Battery (NTB)
Function	None	Alzheimer's Disease Cooperative Study – Activities of Daily Living (ADCS ADL) Scale, Mild Cognitive Impairment (MCI)	Alzheimer's Disease Cooperative Study – Activities of Daily Living (ADCS ADL) Scale; Disability Assessment for Dementia (DAD)
Trial Outcome	Drug-placebo difference in biomarker considered reasonably likely to predict clinical benefit; Reduction in cognitive decline compared to placebo	Drug-placebo difference in a composite outcome plus biomarker outcomes supportive of disease modification (composite differences between drug and placebo should not be due exclusively to cognitive benefits of therapy)	Drug-placebo difference in dual cognitive and functional or global outcomes plus biomarker outcomes supportive of disease modification

amyloid imaging or CSF amyloid measures show that amyloidosis is present. Studies of asymptomatic participants with autosomal dominant mutations often have mixtures of some patients with amyloid abnormalities and some without, offering the possibility of evaluating a DMT as either primary or secondary prevention [73, 74]. Highly sensitive cognitive measures are combined with biomarkers to determine the impact of anti-amyloid therapies [63, 67, 73, 74]. Participants in this stage of preclinical or presymptomatic AD show very mild cognitive decline that may provide an opportunity to establish a drug-placebo difference in cognitive change [75, 76]. Biomarkers reasonably likely to predict future cognitive decline include amyloid imaging and tau imaging. Tau PET correlates better with cognitive decline and MRI measures of brain atrophy and may provide more insight into DM than amyloid measures [77, 78].

Clinical trial design

The most common Phase III design for DMT trials is the randomized, parallel group, placebo controlled, two or more arm, 18–24 month trial. The primary outcome is the drug-placebo difference at trial end on co-primary clinical and functional outcomes or clinical and global outcomes. Biomarker measures typically include MRI volumetrics; amyloid PET (if the agent has a mechanism expected to impact fibrillar amyloid); and CSF Aβ, total tau, and p-tau. Additional biomarkers might be chosen depending on drug MOA and specifics of the trial. Drug-placebo differences at trial end are analyzed for both clinical and biomarker outcomes. Analyses that offer supporting data expected in DM include change in slope of decline, increasing drug-placebo difference over time, and delay to milestones captured in the data (e.g., in a trial of prodromal patients, the percent of patients at each time point who have progressed to a diagnosis of dementia or advanced from a CDR score of 0.5 to a CDR score of 1). These supporting analyses can be affected by symptomatic agents and do not by themselves prove DM. Clinical and biomarker data are expected to be correlated if they are mediated by the same mechanism [79].

The delayed start and staggered withdrawal designs provide evidence of DM without depending on biomarkers. They demonstrate an enduring change in the course of the disease in comparison with a group begun on treatment earlier (in the case of the delayed start design) or withdrawn from therapy (in the case of the staggered withdrawal design) [80–82]. These trials have been difficult to implement and have had limited use in programs attempting to show DM. The switch from placebo to active therapy when a trial is terminated and participants enter an open label extension (all are on active therapy) provides an opportunity for a delayed start observation [83], although the absence of blinding at this stage of the trial could bias the observations. This open-label delayed start analysis could add support to a claim of DM without providing definitive evidence.

Adaptive clinical trial designs use data from the ongoing trial to make decisions about trial conduct. For example, the Dominantly Inherited AD-Treatment Unit (DIAN-TU) uses an adaptive strategy for dose-selection of test agents [84]. Adaptive strategies can

be used for dose, treatment duration, sample size, and entry criteria. The decision structure must be comprehensively pre-specified but adaptive designs have the advantage of responding to the in-trial observations and can save time and resources while optimizing the opportunity to demonstrate a drug-placebo difference [85].

Another resource-saving strategy in clinical trial design and analysis is the incorporation of futility analyses at a time when a sufficient number of patients have been exposed to treatment for a sufficiently long period time to predict the possible outcomes. If the drug-placebo difference at the time of the analysis suggests that the study has a very low possibility of finding a drug-placebo difference at trial conclusion, the trial can be stopped [64, 86]. Futility analyses avoid exposing patients to agents and potential side effects when a positive conclusion of the trial is deemed highly unlikely. Criteria for futility are evolving; they must be liberal enough to insure that potentially viable drugs are not terminated prematurely and conservative enough that trials with very little chance of success are not continued.

The sample size of the trial is determined by the anticipated effect size of the intervention, the variability of the key measurements, and the desired length of the trial. Assuming that a slowing of 20% or more is clinically meaningful for participants and families, the typical trial for a DMT anticipates including 600–1000 subjects per arm and observing them for 18–24 months [87]. Individuals with more severe disease have faster rates of decline. Prodromal patients who are ApoE4 carriers decline more rapidly than those who are not carriers [88]. The decline in the placebo group is critical to assessing the efficacy of the intervention and decline on placebo is a critical determinant of the success of a trial.

LESSONS LEARNED FROM TRIALS OF DMTS

There have been frequent failures in attempts to develop new drugs for AD, and 100% of DMT development programs have failed [6]. Every trial, however, is a learning opportunity and many lessons have been learned that will assist in future drug development [89].

Animal models of AD provide limited evidence of efficacy

Animal models of AD are an important means of investigating efficacy and toxicity in the preclinical state prior to exposing humans to possibly toxic or ineffective compounds. Many of the tg animal models overexpress the amyloid protein leading to cortical plaques similar to those observed in human AD [90]. These genetically engineered animals have abnormalities of amyloid metabolism but generally lack other aspects of human AD; they lack tau or cell death and have limited inflammatory changes [91]. The tg mice have mild cognitive changes but do not develop severe dementia equivalent to the human disease. Many types of therapy have been successful in reducing amyloid abnormalities in these animals and have often lead to improved cognitive performance on tests such as Morris Water Maze or Novel Object Recognition [90]. None of these successes at the preclinical level has predicted success at the human level. The animals serve as important gateways in the drug development process showing that they impact specific pathways; advancing a drug to human testing that did not succeed as expected in animals would be unwise. The models, however, recreate limited aspects of human AD such as amyloidosis and cannot be taken as models of the full spectrum of pathology of human AD or predictors of human benefit [23].

Another concern with regard to animal models is their reproducibility [92]. If an experiment cannot be reproduced within a single model or across related models then its ability to predict human outcomes is suspect. Strain, age, gender, handler behavior, diet, and light conditions may all influence animal behavior. Randomization and sample size are important aspects of animal trial design that have sometimes been ignored [93]. Lack of rigor with regard to these aspects of animal model testing may contribute to the lack of reproducibility both across models and in translating results from animals to humans.

Establish BBB penetration in Phase I

BBB penetration is shown in preclinical studies by the effects of drug on behavioral studies and post-exposure necropsy. Differences between rodent and human BBB function, especially activity of p-gp transporter make extrapolation of animal model results to humans uncertain, requiring demonstration of BBB penetration in Phase I FIH studies [34]. Tarenflurbil is an example of an agent advanced as treatment for AD with *in vivo* activity in animal models but likely low entrance into the CNS in humans [94]. Before candidate agents exit Phase I, investigators should establish BBB penetration, the plasma/CSF ratio, and the relationship of predicted

human brain exposure to concentrations associated with benefit in animal models.

Determine a maximum tolerated dose in Phase I

Dose escalation studies in Phase I and dose refinement studies in Phase II should provide confidence in the dose(s) selected for Phase III. In particular, it is important to establish a MTD whenever possible to ensure that the highest possible doses have been explored. In some cases, occupancy studies may allow conclusions about dosing without an MTD if the receptor is fully occupied at lower doses. In other situations, solubility or physical features may limit the administered dose and the MTD cannot be determined. Beyond these exceptional circumstances, an MTD should be determined. Without an MTD, failure to show a drug-placebo difference in Phase II or Phase III will raise questions about the adequacy of the dose.

The diagnosis of AD should be supported by biomarkers

An important learning is the relatively large number of individuals who have a prodromal AD or AD dementia phenotype but are not amyloid-bearing when studied with amyloid PET [42]. These non-amyloid individuals have suspected non-Alzheimer pathology (SNAP) and are presumed not to have AD. They should be excluded from trials of agents for AD. Table 3 shows the percentage of patients meeting clinical criteria for prodromal AD or mild AD dementia who are amyloid-bearing [42]. Amyloid is more common in those with ApoE genotypes but genetic characterization is insufficient to ensure the presence of amyloid. To be confident that the trial population has AD, amyloid imaging or CSF evidence of the AD Aβ/tau signature should be collected (Fig. 4).

Assure target engagement in Phase II

DM is supported by an impact on "downstream" measures of cell death such as MRI atrophy, CSF tau, or possibly other biomarkers of neuronal degeneration such as neurofilament light chain protein [54]. These downstream consequences can reasonably be expected only if the "upstream" target of the pharmacologic intervention is successful. Target engagement measures will depend on the MOA of the candidate therapy. BACE inhibitors, gamma secretase inhibitors, and gamma secretase modulators will have an effect on amyloid production as measured by stable isotope-labeled kinetics (SILK) [95]. BACE inhibitors will also inhibit BACE activity as measured in the CSF and reflected in sAβPPβ, a by-product of BACE activity; gamma secretase modulators result in Aβ fragments of 15/16 amino acid lengths in the CSF which are not normally present in AD [95–97]. Proof of pharmacology is one goal of Phase II and compounds should not be advanced to Phase III without well documented support for a pharmacologic effect.

Table 3
Amyloid PET findings in patients meeting clinical criteria for prodromal AD or mild AD dementia (stratified by ApoE genotype) [42]

Group	Amyloid Positive	Amyloid Negative
All	61%	39%
All prodromal AD	50%	50%
Prodromal ApoE4 carriers	71%	29%
Prodromal ApoE4 non-carriers	31%	69%
All mild AD dementia	75%	25%
Mild AD dementia ApoE4 carriers	90%	10%
Mild AD dementia ApoE4 non- carriers	58%	42%

Establish a dose-response relationship in Phase II

Dosing approaches in Phase II ideally establish a low dose that is ineffective, one or two mid-range doses that are effective, and a high dose that is not tolerated and not acceptable. A dose-response on clinical or biomarker measures increases confidence in the pharmacology of the molecule. Regulatory agencies usually seek assurance that patients are given the lowest effective dose to ensure that they are not being exposed to unnecessary side effects. Doses established in Phase II inform decisions of which dose should be advanced to Phase III. Drug formulation decisions should be completed in Phase II prior to Phase III.

Collect multiple biomarkers to assess outcomes

Knowledge of the neurobiology of AD is incomplete. Systems biology studies demonstrate that AD biology is complex [98] and biomarkers provide limited windows onto this complex and ill-understood disease. Although working models of the order of events in AD have been constructed, none have been proven and none have guided successful DMT development. Agnostic approaches to biomarkers (e.g., amyloid; tau, neurodegeneration; A/T/N) are used to

acknowledge the exploratory nature of our biomarker documentation of drug effects [99]. To support DM as the outcome of a therapy, trial sponsors should collect A/T/N biomarker data, emerging biomarkers, and biomarkers specifically linked to the mechanism of the intervention to gain a comprehensive view of the impact of treatment.

Recruitment is a major challenge

Trial recruitment is a difficult process and each population—cognitively normal at-risk participants for prevention trials, minimally impaired biomarker positive participants for prodromal AD trials, and cognitive and functionally impaired participants for AD dementia trials—have unique requirements for identification, recruitment, informed consent, and retention in the trial. There are too few highly functioning trial sites in the world. The world's populations are generally poorly educated about clinical trials and often have few opportunities to participate. Many trials spend more time in the recruitment phase of the trial than in the drug exposure phase. Slow recruitment slows the cycle time of trials and increases their cost. Many AD-concerned organizations are constructing responses to this challenge. The Global Alzheimer Platform (GAP) network of trial sites in the US and the European Prevention of Alzheimer's Disease (EPAD) initiatives are among the leaders of the attempt to reduce recruitment times and accelerate trials [100, 101].

Global trials have greater variability

One response to slow recruitment is to include many trial sites with each site recruiting only a few participants to the trial. In most trials, each site is expected to contribute 6–12 participants, but many sites contribute only 1 or 2 participants. This amplifies "noise" in the data and decreases the ability to demonstrate a drug-placebo difference.

Globalization of trials creates another set of challenges. Sites distributed around the world are culturally and linguistically diverse, have different standards of health care, and include participants with different histories of nutrition and levels of education. Trial sites are highly variable in terms of experience, expertise, training, and infrastructure. Local hospital and university institutional review boards (IRBs) may have limited experience with hosting and reviewing AD trials [102]. Global sites impose challenges in terms of drug manufacturing and distribution, supply lines, biomarker collection, laboratory availability, and data collection and quality assurance. The result of this complexity is that populations recruited into trials from around the world vary in terms of age, education, genotype, and other clinical characteristics, and they progress somewhat differently in clinical trials [103, 104]. North America and Western European trial populations are similar and results are likely to be most interpretable if these populations comprise the majority of the study population.

Efficacy and safety data are needed on all populations where the agents will be marketed; smaller trials in local populations may be the best way to address these needs.

Comprehensive trial networks are needed to conduct AD trials

Conducting clinical trials is demanding and requires expertise, commitment, and infrastructure. Some academic medical centers support trials while others do not, industry sponsors support trials but tend not to support trial infrastructure. In the US, the National Center for Advancing Translational Sciences (NCATS) sponsors Clinical and Translational Science Awards (CTSAs) to provide trial infrastructure in major university medical centers [105]. Trial networks are currently re-created for each trial and raters are re-trained on the same outcomes for each trial. Each institution often has its own IRB for reviewing trials. Legal review of contracts further slows trial initiation. Construction of a highly efficient trial network with standing non-redundant training, and a central IRB are goals of GAP and EPAD [100, 101].

Negative trials may indicate an ineffective drug or a failed trial

The failure to show a drug-placebo difference at the end of a trial may be due to lack of efficacy of the candidate therapy or flawed conduct of the clinical trial. Table 4 summarizes the reasons for negative outcomes in trials. Drug-related reasons for negative trials include lack of efficacy and excessive toxicity [106]. In some cases, the dose range has not been adequately explored in early drug development and a negative trial opens the question of whether the agent might have been efficacious at higher doses. Such agents must return to Phase I for dose escalation trials and sponsors rarely have an interest in pursuing this alternative. Trial-related reasons for failed tri-

als of DMTs include lack of decline in the placebo group, enrollment of non-AD patients, and excessive measurement variability.

Placebo decline determines drug-placebo difference

Successful DMTs will slow the course of decline in AD. Slowing of decline is established by contrasting the decline in the active treatment group with the trajectory of the placebo group. The placebo trajectory will determine the drug-placebo difference at end of trial. The rate of decline of the placebo group is a crucial consideration in understanding the treatment effect. Placebo groups with SNAP patients do no decline as rapidly as those with confirmed AD, emphasizing the importance of confirming the diagnosis of AD in trial participants [107]. Slow decline in the placebo group will minimize the drug-placebo difference and the agent will appear less efficacious than when compared with a more rapidly declining group. Similarly, an unusually rapidly declining placebo group may lead to an overestimation of drug efficacy since the drug-placebo difference will be exaggerated and this may not be reproduced in a later trial. A meta-analysis of placebo decline showed that patients with mild AD are expected to decline 5.6 points on the ADAS-cog or 3 points on the Mini-Mental State Examination in 18 months [108]. This figure is based on trials that included patients without biologically confirmed AD and may underestimate the decline in those confirmed with amyloid imaging or CSF studies to have AD.

Table 4
Reasons for failure to show a drug-placebo difference at the end of a clinical trial of a disease-modifying agent. AD, Alzheimer's disease

Drug-related
• Lack of efficacy of the agent
• Inappropriately low dosing of an effective agent
• Excessive toxicity or lack of tolerability leading to high discontinuation rates in the active treatment arms
• Excessive toxicity or lack of tolerability leading to early termination of the trial
Trial-related
• Lack of decline in the placebo group
• Recruitment of non-AD patients into trials requiring an AD substrate for drug benefit to occur
• Excessive measurement variability
• Lack of measurable effect of active comparator drugs (if available)

Phase II subgroup analyses do not provide guidance for Phase III

Negative trials are often analyzed to detect treatment-responsive subgroups that can be exploited in future trials. This approach entails substantial risk of being misled by spurious trial specific results. Subgroups are not subject to the same recruitment or randomization as the original group, the sample sizes of subgroups are often small leading to underpowered results, and the outcome measures are typically not optimized for a specific subgroup. Basing a Phase III program on a subgroup analysis of a Phase II trial with a negative outcome has usually resulted in a negative Phase III trial.

Table 5
Questions to ask to determine how much confidence can be placed in a subgroup analysis [109–111]

Guide: Questions to Ask of Subgroup Claims	Supportive of Subgroup Claim if "Yes"
Design	
Was the subgroup variable a baseline characteristic?	
Was the subgroup variable a stratification factor at randomization?	
Was the subgroup hypothesis specified a priori?	
Was the subgroup analysis one of a small number of subgroup hypotheses tested ($\leq$5)?	
Analysis	
Can chance explain the subgroup difference?	
Was the test of interaction significant ($p < 0.05$)?	
Was the significant interaction effect independent, if there were multiple significant interactions?	
Context	
Was the direction of the subgroup effect correctly pre-specified?	
Was the subgroup effect consistent with evidence from previous related studies?	
Was the subgroup effect consistent across related outcomes?	
Was there indirect evidence to support the apparent subgroup effect – for example, biological rationale, laboratory tests, animal studies?	
Systematic reviews	
Is the subgroup difference suggested by comparisons within rather than between studies?	

To reduce the risk of being misled, one can apply guidelines for interpretation of Phase II subgroup analyses. Table 5 shows the principal recommendations for subgroup analysis [109–111]. Subgroup analyses suggesting benefit in one group of patients require conducting a Phase II trial for this subgroup to gain additional confidence in this treatment approach.

BLUEPRINT OF A DEVELOPMENT PROGRAM FOR A DMT

This primer of DMT trials plus the lessons learned from negative trials suggest a blueprint for future trials of DMTs. The key elements of success for a DMT development program include:

- Comprehensive understanding of target biology
- Selective, potent agents impacting a key element of AD biology leading to cell death
- Disciplined conduct of a drug development program organized around a TPP
- Success in preclinical models of AD
- Acceptable ADME and toxicity in preclinical studies
- Acceptable ADME and toxicity in FIH studies
- BBB penetration demonstrated with relevant extrapolated brain exposures achieved in Phase I
- MTD established in Phase I
- Use of biomarkers in Phase II and III to establish accurate diagnosis of AD
- POC established in Phase II with target engagement and proof-of-pharmacology
- Dose-response shown in Phase II
- Trials implemented in high functioning trial network
- Globalization-dependent variability minimized in Phases II and III
- Demonstration of robust clinical and correlated DM-type biomarker response in Phase III
- Report Phase II and III trials using CONSORT criteria
- Continued assessment of safety and clinical utility after market introduction

SUMMARY

Development of DMTs for AD is a difficult, long, and expensive process. No development program has yet succeeded. A systematic approach to drug development advancing the scientific understanding of the candidate molecule from preclinical studies through Phases I, II, and III of clinical trials can increase the probability of success and de-risk development programs. Biomarkers for diagnosis, target engagement and proof-of-pharmacology, outcome assessment, and side effect monitoring assist in drug development. Excellent conduct of trials and awareness of the trial pitfalls are critical to development success. New therapeutic targets such as tau-related processes and the use of combination therapies may enhance the chances of successful DMT development. Quality development and trial strategies for drugs that are potent, selective, and impactful on the biology of AD are necessary to bring urgently needed new treatments to patients with AD and those at risk for the disease.

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Targeting Alzheimer's Disease at the Right Time and the Right Place: Validation of a Personalized Approach to Diagnosis and Treatment

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Abstract. Cautious optimism is appropriate for a near future (five years) time frame for a number of drugs acting on the different pathophysiological components of Alzheimer's disease (amyloid deposition, tau hyperphosphorylation, neuroinflammation, vascular changes, to name the most important known so far). Since the relative weight of these components will be different between individuals and will even change over time for each individual, a 'one drug fit for all' approach is no longer defensible. Precision medicine using biomarkers in the diagnosis and treatment of Alzheimer's disease is the new strategy.

Keywords: Alzheimer's disease, biomarkers, brain imaging, database analysis, diagnosis, human volunteer cohorts, precision medicine, translational research, treatment

MISE-EN-CONTEXTE

The traditional treatment approach: Monotherapy for all patients with the Alzheimer's disease phenotype

The renaissance of interest for Alzheimer's disease (AD) started in the 1970 s when a cholinergic deficit was discovered, leading to a transmitter-replacement therapy using cholinesterase inhibitors. A modest but clinically meaningful improvement was demonstrated in randomized clinical trials (RCTs) and in clinical practice for mild to severe stages of dementia. The NMDA-receptor antagonist memantine was then found to improve patients in moderate to severe stages of dementia. Proof of additive benefit from the two classes of drugs is still equivocal. These drugs proved to be relatively safe considering the age and co-morbidity of most users [1].

The next phase of AD research centered on amyloid-β $(Aβ)_{42}$, in late onset sporadic as well as early familial AD. At the dementia stage of AD, no RCTs targeting amyloid have been successful to this date, despite multiple attempts using active or passive immunization, BACE-inhibitors, and γ-secretase inhibitors, in patients known to have amyloid pathology using positron emission tomography (PET) scanning or cerebrospinal fluid (CSF)

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examination. The most encouraging study is a Phase Ib RCT using aducanumab in mild AD which showed a dose-related reduction in amyloid load using PET as well as clinical stability [2].

A first attempt at treating the tau pathology associated with AD using an orally administered tau aggregation inhibitor led to equivocal results in mild to moderate dementia [3]. Other studies are being initiated using monoclonal antibodies [4]. Tau pathology is now recognized as a key driver of disease progression in AD, and strategies are being developed to accelerate drug development from animal models, such as antibodies against the proximal N-terminal domain tau 6–18 in 3XTg-AD mice [5], to human RCTs [6].

Finally, attempts at reducing the levels of neuroinflammation using non-steroidal anti-inflammatory drugs such as naproxen have failed to modify cognitive decline in persons at risk or with dementia due to AD [7].

New diagnostic criteria using biomarkers

The clinical progression of AD is linked to specific neuropathological features, such as extracellular deposition of Aβ plaques, intracellular inclusions of tau protein in neurofibrillary tangles, and neuronal degeneration. The discovery and advance of disease biomarkers over the last decade have significantly advanced our understanding of the dynamic pathophysiological changes underlying AD and have allowed the detection of AD pathophysiology *in vivo* [8]. Given that the presence of AD pathophysiology has been found across a broad clinical spectrum including individuals asymptomatic and with mild cognitive symptoms, biomarkers now play an important role in characterizing the trajectory of AD pathophysiology and have been incorporated in the AD diagnostic research criteria [9–12]. These diagnostic research criterions recognize that the coexistence of abnormal Aβ and tau biomarkers better identify the preclinical and mild cognitive impairment (MCI) individuals who will progress to dementia over relatively short time frames of three to five years. These concepts also apply to well to early onset familial AD [13].

Such advances in understanding the natural history of AD have been made possible through concerted international efforts at pooling the database of research cohorts. Indeed, analysis of the data obtained from cohorts of volunteers undergoing periodic clinical, neuropsychological, and biomarkers assessments have led to multiple publications, which further enhance our knowledge of the AD process. The Alzheimer's Disease Neuroimaging Initiative (ADNI) is the best known of these cohorts, and the open access of these data to scientists is considered as a model for science as a whole. Access to the Dominantly Inherited Alzheimer Network (DIAN) database is more restrictive because of specific ethical considerations due to the genetic status of the participants.

The identification of AD biomarkers crossing pathological threshold in cognitively normal individuals has led to the conceptual framework of a preclinical stage in AD [14]. This operational definition of preclinical AD has made possible ongoing RCTs in individuals by the Dominantly Inherited Alzheimer Network Trials Unit (DIAN-TU) [15] and the Anti-Amyloid treatment in Asymptomatic AD (A4) study [16], given that early intervention may offer the greatest chance of treatment success.

New concept of individualizing the underlying pathophysiological components of AD

Based on histopathological and genetic evidence, fibrillar Aβ, the main constituent of Aβ plaques, has been postulated as the major driving force leading to AD dementia (amyloid cascade hypothesis). According to this hypothesis, all the resulting pathological processes are due to an imbalance between Aβ production and clearance, which would then potentiate the spread of tauopathy, leading to neurodegeneration and cognitive decline. However, the lack of consistent association between Aβ and clinical progression, and the fact that amyloid pathology has been found in cognitively normal elderly individuals challenge the Aβ hypothesis in its original form. For example, tau pathology has been reported in the brains of non-demented subjects in the transentorhinal, limbic, and basal temporal regions [17, 18], while studies have shown that either Aβ or neurodegeneration may be the first biomarker abnormality in preclinical AD [19]. Therefore, alternative pathways of the AD pathophysiology independent of Aβ have been suggested [20, 21].

An unbiased biomarker classification system, A/T/N, which avoids the assumptions of the temporal ordering of AD biomarkers, has been proposed [22]. In this classification system where each biomarker category is binarized as either positive or negative, "A" represents Aβ biomarkers using amyloid PET or CSF $A\beta_{42}$, "T" represents tau biomarkers

using CSF p-tau or tau PET, and "N" represents neurodegeneration biomarkers using CSF p-tau, structural magnetic resonance imaging (MRI), or [^{18}F]fluorodeoxyglucose PET (FDG). This descriptive classification aims to organize the multi-modality biomarker results at the individual person level in a way that is easy to adopt and interpret. Other brain pathological processes have been postulated as natural candidates to integrate this unbiased system. Studies under way are measuring simultaneously the amyloid, tau, and neuroinflammation in individuals, with follow-up over time to test the hypothesis that the coexistence of the brain pathological factors may accelerates AD clinical manifestations (*vide infra*). If confirmed, the A/T/N classification of individuals may be broadened to A/T/N/NI, where "NI" represents neuroinflammation biomarkers. Another important biomarker candidate is the presence of concomitant cerebrovascular disease. Indeed, results from DIAN cohort have already suggested white matter hyperintensities as a core pathological feature of autosomal AD [23], and a multifactorial causal model analysis using ADNI demonstrated the importance of vascular dysregulation as an important initial pathologic event leading to late onset AD [24].

CONTRIBUTIONS OF THE MCGILL CENTER FOR STUDIES IN AGING TO THE FIELD

Our main research activities over the past three years are summarized in Table 1.

Translational research from animal models, human postmortem tissues, and in vivo human volunteers

One of our research strategies has been a translational approach to the study of cerebral biomarker changes with age and in AD participants, as well as animal models. For example, using the McGill-R-Thy1-APP rat model, we were able to simultaneously demonstrate changes in MRI, CSF, PET, and brain tissue levels of $A\beta_{42}$ over time [25, 26]. The less aggressive Aβ progression in these transgenic rats makes this model more similar to the insidious progression of late onset human sporadic AD. Also, the relatively large brain size of these rats makes possible the identification of specific brain structures using PET. It is important to mention that, using a CSF cisternal collection technique, we were able to perform the first longitudinal study of CSF at multiple time points *in vivo* without causing any harm to the animals. Additionally, one of our interests is to bridge the knowledge from these studies in animals to human volunteers. In this regard, over the past few years, we have also been able to bridge studies in animal models [27] and human volunteers for cholinergic denervation [28], and similarly for glutamate mGluR5 receptors studies [29]. We also have successfully begun working with PET radiotracers demonstrating tau binding with high specificity relative to monoamine oxidase [30], and we have proven that [^{18}F]FDG depicts astrocytic activity in addition to synaptic neuronal activity [31].

Prospective study of age-associated biomarkers

Cortical thickness using MRI has been studied in a cohort of cognitively normal (CN) persons between ages of 40 and 80, at two-year intervals. In this cohort, we have genetically and morphologically characterized familiarity deficits as an early cognitive maker for individuals at risk for AD [32, 33].

We are currently simultaneously studying over time amyloid, tau, and neuroinflammation using PET imaging. This new cohort includes cognitively normal older persons, MCI due to AD, and mild dementia due to AD in early onset familial cases as well as late onset sporadic cases, as a first step to a personalized approach to treatment targeting multiple pathological pathways (*vide infra*).

Research using large database

Another successful approach has been the study of the interaction between $A\beta_{42}$ and tau biomarkers in AD, using the ADNI database. We found that the synergy of Aβ and hyperphosphorylated tau, rather than their individual effects, drives metabolic decline in preclinical AD (Fig. 1) [34], and that this synergy also predicts progression from MCI to dementia [35]. Other uses of ADNI include linking immune cascades and cerebral amyloidosis using epistasis analysis [36], finding biomarker characteristics of rapidly progressive AD [37] and of CN individuals with ventriculomegaly [38], and a correlation between early neuropsychiatric symptoms and hypometabolism in preclinical AD [39].

Another important database for our analysis in DIAN. A first study looking at suicidality risk in carriers and non-carriers suggests a similar risk, and a prediction model using neuropsychiatric symptoms and neuroimaging/CSF biomarkers is under study.

Table 1
Main research activities in the MCSA over the past three years

Study subjects (Sample size)	Study design	Biomarkers	Main outcome measured	Scientific Contribution	Reference
Translational research from animal models, human post-mortem tissues and in vivo human volunteers					
5 bvFTD 10 CN	Case-control	[^{11}C]ABP688 PET	Glutamatergic abnormalities	First *in vivo* report of decreased availability of mGluR5 in FTD.	[29]
11 wild-type rats	Drug challenge	[^{11}C]ABP688 PET	Glutamatergic binding sites	Supports that mGluR5 availability is sensitive to extracellular glutamate.	[54]
5 CN 7AD	Case-control	[^{18}F]FEOBV PET	Cholinergic denervation	First quantification of brain cholinergic denervation in AD patients.	[28]
5 MCI 2 AD 1 PSP	Drug challenge	[^{18}F]THK5351 PET	MAO-B availability	First *in vivo* study showing that [^{18}F]THK5351 highly depict MAO-B availability, rather than tau deposition, in the brain.	[30]
13 wild-type, 13 McGill-R-Thy1-APP	Longitudinal observational cohort	[^{18}F]Florbetapir and [^{18}F]FDG PET. CSF Aβ and tau. MRI.	Biomarkers change over time	Suggests that Aβ itself is sufficient to impose focal memory circuits dysfunction	[26]
10 wild-type rats	Drug challenge	[^{18}F]FDG PET	[^{18}F]FDG availability	First study showing strong evidence that astrocytes contribute significantly to the [^{18}F]FDG signal.	[31]
Research using large database					
196 CN 324 MCI 70 AD	Epistasis analysis	[^{18}F]Florbetapir PET. CSF Aβ and tau.	Fibrillary amyloid-β	Genetic components linking the immune system and brain amyloidosis.	[36]
120 CN	Prospective longitudinal observation	[^{18}F]Florbetapir and [^{18}F]FDG PET. CSF Aβ and tau.	Changes in glucose metabolism	First study showing the synergy between Aβ and tau drives metabolic decline in preclinical AD	[34]
314 MCI	Case-control	[^{18}F]Florbetapir and [^{18}F]FDG PET. CSF Aβ and tau.	Progression to dementia	First study showing that a synergy between Aβ and tau determines the progression to dementia	[35]
312 mild AD	Prospective longitudinal observation	[^{18}F]Florbetapir and [^{18}F]FDG PET. CSF Aβ and tau. MRI.	Rapid progression to dementia	Identification of the biomarkers best associated with rapid progression to dementia	[37]
115 CN	Prospective longitudinal observation	[^{18}F]Florbetapir and [^{18}F]FDG PET. CSF tau. Neuropsychiatric symptoms	Changes in glucose metabolism	Supports that neuropsychiatric symptoms constitute an early clinical manifestation of AD.	[39]
425 CN	Prospective longitudinal observation	[^{18}F]Florbetapir and [^{18}F]FDG PET. CSF Aβ and tau. MRI (ventriculomegaly)	Biomarkers change over time	Ventriculomegaly might be an early imaging signature of AD and/or normal pressure hydrocephalus.	[38]
Prospective study of age-associated biomarkers					
81 CN	Prospective longitudinal observation	APOE ε4	Familiarity performance	APOE ε4 is associated with a reduction in familiarity in the absence of other cognitive deficits.	[32]
81 CN	Prospective longitudinal observation	Structural MRI	Familiarity performance	Familiarity is associated with the cortical volumes in APOE ε4 carriers.	[33]

(continued)

Table 1
(*Continued*)

Study subjects (Sample size)	Study design	Biomarkers	Main outcome measured	Scientific Contribution	Reference
New analytical techniques, animal model platforms, and software development					
APPJ20/T64 mice and McGill-R-Thy1-APP rat	Animal model platform for AD	Brain imaging	Changes in PET imaging over time	Development of a platform for AD research using PET imaging and transgenic models	[25]
1,536 participants	Software development and validation	Computational cognitive battery	Cognitive decline	Development of a free platform for adults aged 40–90 to engage in cognitive training	[42]
273 samples	Software development and validation	Brain imaging	Voxel-wise changes brain imaging	A novel computational tool able to perform complex voxel-wise statistical in humans and animals	[43]
273 MCIs	Machine learning	[^{18}F]Florbetapir PET	Progression to dementia	The algorithm to predict incipient dementia with accuracy outperforming existing algorithms	[41]

Abbreviations: AD: Alzheimer's disease; APOE: apolipoprotein E; APP: amyloid precursor protein; bvFTD: behavioral version of fronto-temporal dementia; CN: cognitively normal; CSF: cerebro-spinal fluid; FDG: fluroro-deoxy-glucose; MAO-B: monoamine oxidase type B; MCI: mild cognitive impairment; MRI: magnetic resonance imaging; PET: positron emission tomography; PSP: progressive supra-nuclear palsy.

New analytical techniques and software development

The accuracy and optimization of neuroimaging methodological techniques is a special interest of our Center. Various different methods of analysis using neuroimaging modalities such as PET, MRI, and fMRI have been explored. For example, we have compared the accuracy of two widely used automated protocols (FreeSurfer and FSL) for MRI brain segmentation against the gold standard manual segmentation [40]. Using single Aβ PET information, we develop a novel analytical algorithm based on machine learning that outperformed all the existing algorithms using multiple biomarkers [41].

Website and software development in our Center has a significant impact. The Prevention Of Neurodegenerative Disease in Everyone at Risk (P.O.N.D.E.R) program was conceptualized to offer a free online platform for adults to participate in cognitive training and to be a large-scale tool to identify persons showing early signs of cognitive decline. 1,536 individuals have already signed up on the program's website (http://ponder.mcgill.ca) and underwent a standardized computerized battery assessing memory, executive function, attention, constructive abilities orientation, problem solving, language, and perception [42]. Additionally, in the neuroimaging field, our associates have developed a singular computational framework that allows us to perform complex voxel-wise statistical operations with multiple scalar variables and image modalities at every brain voxel in humans and animal models [43].

Ethical issues in biomarker research

The ethical aspects associated with AD diagnosis and treatments have been a constant topic of interest in our center since its inception. More specifically, our research team have addressed ethical issues associated with the use of biomarkers in asymptomatic persons, very early disease diagnosis, and possibility of access to new treatments [44, 45]. Additionally, participation in the Ethical, Legal and Social Aspects (ELSI) committee of the Canadian Consortium of Neurodegeneration in Aging (CCNA) is a core part of our activities. This has facilitated the establishment of a trans-national scientific and ethics review for dementia research [46].

Education and knowledge transfer

One of the main aspects of our educational activities is the formation of the new generation of health professionals and researchers in AD. Every year, undergraduate, master, and doctoral students begin working on our project under the supervision of our members. In addition, our center receives a high flow

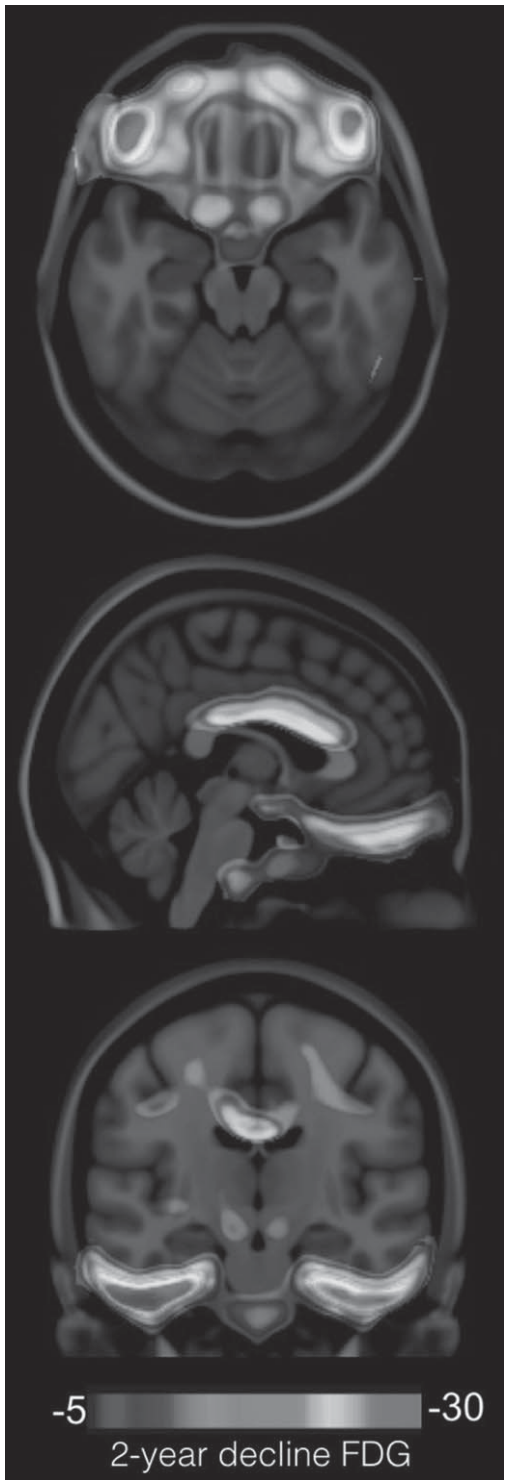

Fig. 1. Brain regions vulnerable to the synergy between Aβ and tau in cognitively normal persons. The parametric map, overlaid in a structural MRI, revealed regions where 2-year [^{18}F]FDG metabolic decline was associated with the synergistic effect between Aβ and tau in cognitively normal elderly individuals.

of international students and visiting scholars who come as a complementary part of their studies to learn and share knowledge with our students and members. Seminars and lectures targeting AD relatives and the general public are also part of our work.

NEW DIRECTIONS

Drug discovery

The most recent review of the AD drug development pipeline demonstrated insufficient drug discovery activity to supply new agents for testing in RCTs [47], a concern previously noticed and blamed on the nosology and complexity of the biological mechanisms of AD (particularly in late onset with multiple co-morbidities), the low success of drugs for this condition, slow recruitment, and low retention for large scale RCTs in older persons [48]. Promising strategies to overcome these difficulties include sharing placebo groups, as currently done in the DIAN-TU RCTs, futility analysis, adaptive designs, patients, and volunteer registries. Another approach is to learn from the past RCTs about biological sub-groups of individuals responding to treatment. A good example is the ApoE4/4 carriers in the tramiprosate studies, which showed clinical stability over time compared to the overall group and the controls [49].

Biomarkers as a contribution to precision medicine in AD

Precision medicine is a concept which describes the biomarker guided identification of specific biological and molecular pathophysiologies in an individual with the aim of applying a personalized preventive or therapeutic approach which will more accurately target the particular biological dysfunction [50]. In a multifactorial disease such as AD, this investigation and therapeutic strategy is especially important as compared to the traditional "one pathophysiology fits all" approach. In this respect, the advancement of biomarkers research such as genetics, neuroimaging, and biological fluids, is expected to play an important role in decoding the specific pathophysiological alterations in each individual at risk for AD.

In line with precision medicine in AD where biomarker guided customized interventions may offer the best chance of therapeutic success, both

environmental and genetic factors have been included in recent clinical trials to enrich the study population with the highest risk of developing AD as soon as possible. For example, in the Alzheimer's Prevention Initiative (API) autosomal dominant AD trial, preclinical PSEN1 E280A mutation carriers have been recruited to study the efficacy and safety of crenezumab, while in the DIAN-TU trial, investigational drugs (gantenerumab and solanezumab) are being tested in individuals who either are known to carry a mutation, or are at risk due to a positive family history for a known AD-causing mutation in a parent or sibling, to evaluate these drugs impact on biomarker changes over time, and potentially demonstrating cognitive efficacy. In the API Generation study, CN healthy older adults who are at high-risk of developing AD based on their age (60–75 years) and genetic background (homozygous APOE4) are being recruited to study the cognitive efficacy of the active amyloid immunotherapy CAD106 or the beta secretase inhibitor 1 (BACE1) inhibitor CNP520 in preventing or delaying AD.

In these and future RCT targeting various pathophysiological factors at play in AD, biomarkers will play a major role in defining the populations to treat, and quantify the treatment response. With a bit of luck, we will be able to select the new drugs for the right patient at the right stage of disease, using individual biomarker profile.

Global perspective on AD prevention and treatment

From a global perspective, we need to learn from the success of therapies against cancer and infectious diseases, from a RCT design perspective [51] as well from a drug access perspective, when new therapies will have been demonstrated to be safe and effective [52]. Costs of earlier diagnosis are already being studied [53]. National dementia plans will have to adapt to new knowledge about early diagnosis and treatment as quickly as feasible once reliable scientific information has been disseminated.

CONCLUSIONS

Cautious optimism is appropriate for a near future (five years) time frame for a number of drugs acting on the different pathophysiological components of AD (amyloid deposition, tau hyperphosphorylation, neuroinflammation, vascular changes, to name the most important known so far). Since the relative weight of these components will be different between individuals and will even change over time for each individual, a 'one drug fit for all' approach is no longer defensible. Precision medicine using biomarkers in the diagnosis and treatment of AD is the new strategy.

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Lost in Translation? Finding Our Way To Effective Alzheimer's Disease Therapies

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Abstract. Efforts over the past two decades to develop effective disease-modifying treatments for Alzheimer's disease have been disappointing, while parallel efforts in another chronic neurologic disease, multiple sclerosis, have been remarkably productive. In an effort to advance development of therapeutics for Alzheimer's disease, these two fields are contrasted in terms of the utility of animal models, definition of study populations, and utility of biomarkers. Possible solutions are suggested, and the review concludes with description of some active peer-reviewed, publicly funded clinical studies which address some of the identified weaknesses in past clinical trials for age-related dementia.

Keywords: Alzheimer's disease, animal models, clinical trials

INTRODUCTION

The inability to demonstrate disease-modifying effects in Alzheimer's disease (AD) has been a great frustration for patients, clinicians, and investigators. These repeated failures may be due to over-reliance on invalid hypotheses, an unfortunate choice of specific interventions, problems with clinical trial design, or myriad other explanations. Debates on these points are rarely conclusive, in part because there is not a positive outcome to contrast with all of the negative results and make the case for paradigm change in hypothesis or in trial design. Since failure is the norm in AD therapeutics research, we will need to look to other fields to find examples of success to guide us. We will consequently proceed with a brief review of a representative failed clinical trial in AD, and then consider the elements of success in a more productive field (multiple sclerosis therapeutics). We will then conclude with some thoughts about where the field should move in the future.

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A REPRESENTATIVE CLINICAL TRIAL: THE NIA-ADCS DHA TRIAL TO SLOW THE PROGRESSION OF AD [1]

The decision to conduct a multi-center trial of an omega 3 fatty acid for AD, at a cost of approximately ten million NIH dollars, was the result of a long, thoughtful, systematic deliberation by the Alzheimer's Disease Cooperative Study (ADCS) leadership and Steering Committee. The process started with submission of a protocol synopsis to the Project Selection Committee, which selected the study for presentation at a meeting of the Steering Committee, comprised of approximately 35 AD clinical trial experts from the various sites. After the Steering Committee selected the omega 3 trial for further development, the protocol was then presented to

an independent Scientific Advisory Board. After the scientific advisors also endorsed the protocol for further development, the project was included as one of five clinical trials in the competitive renewal application for the ADCS and went to NIH study section. The study section recommended funding the trial. In other words, this trial was initiated only after unusually thorough peer review, so retrospective consideration of the final outcome cannot point to lack of due diligence in explaining the failure to find therapeutic benefit with this strategy. The rationale for the study was two-fold: 1) abundant epidemiologic data indicated that dietary consumption of fish and/or omega 3 fatty acids was associated with a lower incidence of dementia [2] and 2) studies in transgenic mouse models of AD demonstrated that supplementation of the omega 3 fatty acid docosahexaenoic acid (DHA) produced anti-amyloid and neuroprotectant effects [3, 4]. The animal studies chose DHA as the specific omega 3 fatty acid for supplementation because DHA is the most abundant polyunsaturated fatty acid in the brain and is enriched in synaptic fractions, whereas eicosapentaenoic acid (EPA), the other omega 3 fatty acid thought to be responsible for the health benefits of fish consumption, is found at only very low concentrations in brain tissue.

The DHA trial was enrolled on time, compliance was excellent, DHA was well tolerated, and retention was within the anticipated range [1]. Unfortunately, however, the DHA-treated participants progressed at the same rate as the placebo-treated participants, and this negative result was reported in 2010 [1]. A pre-specified sub-group analysis showed a statistically significant effect of DHA treatment upon one of the two primary outcome measures in the ApoE4 non-carriers, which comprised about half of the overall study population. However, there was no effect of DHA treatment in the non-carriers on the other co-primary outcome, nor on any of the secondary outcome measures, so the isolated positive finding with one outcome measure in E4 non-carriers was reported but not emphasized in the final publication [1].

Reasons for failure

In retrospect, some of the possible reasons for the failure of this trial to find a therapeutic benefit of DHA include:

1) *Dependence on animal models that are not predictive of clinical outcomes*. The APP mice used in the preclinical studies [3, 4] have been used to screen hundreds of candidate therapies, many of which have moved on to clinical trials, and none of which have been shown to have robust clinical effects.

2) *Insufficient attention to the study population*. Epidemiologic data pre-dating the trial suggested that the brain health benefits of omega 3 fatty acids may be more evident in E4 non-carriers compared to carriers [5, 6], so confining a trial to non-carriers may have yielded a different result. Subsequent investigations have explored the possibility that E4 carriers may be unable to benefit from DHA because of genotype-associated impairment in DHA uptake [7]. Natural history studies and other clinical trials also suggest that any intervention targeting amyloid-β (Aβ) may need to be initiated early in the disease course, before the onset of overt dementia, in order to achieve a therapeutic benefit, so the mild to moderate AD population targeted in this trial may have been too far along in the disease course to benefit from any anti-amyloid intervention. In fact, a *post hoc* analysis of another omega 3 fatty acid trial in AD suggested the benefit was confined to the most mildly affected individuals [8], so a focus on early AD or MCI might have increased the chance of seeing a therapeutic effect.

3) *Sacrificing the rationale for the study to the practicalities of clinical trial design.* The rationale for the DHA study was the observation that omega 3 fatty acid intake was associated with a lower risk of AD, but the clinical trial evaluated the effects of omega 3 on disease progression, rather than on disease onset. This decision was a practical concession to the fact that prevention studies require thousands of subjects followed for several years, while a treatment trial can be powered to detect an effect with hundreds of subjects followed for 18 months. However, we have several lines of evidence to suggest that mechanisms of disease underlying AD initiation and AD progression are not identical. For example, ApoE genotype seems to be important for disease initiation but not necessarily for disease progression. The ADNI [9] and DIAN [10] biomarker data illustrate that different phases of AD initiation and progression are marked by shifts in different biomarkers, suggesting that the mechanisms of disease also change over the course of the disease. This extrapolation from evidence for *prevention* effects to testing of *treatment* effects has also been applied in other trials of NSAIDs [11], statins [12], and homocysteine-lowering vitamins [13] for AD, with similar negative outcomes in each of those trials.

Other transitions from epidemiologic observation to trial design may also exert confounding effects.

For example, the use of pure DHA rather than mixed omega 3 fatty acids in the DHA trial did not directly follow from the epidemiology, which evaluated consumption of omega 3 fatty acids as they exist in the diet, with DHA and EPA in combination. Since EPA may have greater effects than DHA on some endpoints like vascular health, the decision to focus on a single omega 3 fatty acid may have compromised the ability to find a treatment effect.

4) *Absence of surrogate outcome measures for a "proof of concept" clinical trial as an intermediate step between transgenic mouse studies and full-scale clinical trial*. This practice of leaping from animal studies to full-scale trials has been repeated many times in the effort to develop AD therapeutics and will likely continue until a paradigm for evaluating candidate therapies in smaller cohorts is shown to predict effects in full-scale trials. The protocol for serial sampling of radio-labelled cerebrospinal fluid (CSF) Aβ via lumbar drain in human subjects is a promising example of an informative "proof of concept" design [14] which may permit rational "go-no go" decisions [15] in the evaluation of experimental agents for AD, but this method is technically demanding and there is ample room for additional outcome measures for this purpose.

A REPRESENTATIVE SUCCESS STORY: THERAPY DEVELOPMENT IN MULTIPLE SCLEROSIS

The repeated successes in multiple sclerosis (MS) over the last 20 years stand in sharp contrast to the repeated failures in AD drug development during the same time frame. It may be instructive to attend to elements that may have promoted success in the MS field, contrasting them with the four points listed above:

1) *Identification of an animal model predictive of clinical effects*. Many (although not all) disease-modifying drugs for MS were initially evaluated in the experimental allergic encephalomyelitis (EAE) mouse model of MS. While the EAE model is by no means a perfect model of the human disease, it is clearly a useful model for drug development [16]. The arguments about the relative value of different animal models in the AD field, including the argument that no AD animal models are valid, miss the point illustrated by EAE and the development of MS drugs: animal models need not be perfect in order to predict clinical outcomes.

2) *Careful attention to definition of study population.* The landmark trial of beta-interferon, the first disease-modifying drug approved for MS, was at the time novel in its restriction of the study population to patients with a particular phenotype (relapsing-remitting) and an established level of disease activity (two relapses within the last year) [17]. Once proven in the beta-interferon study, this aspect of trial design became standard in MS drug development, leading to the approval of 15 disease-modifying drugs for relapsing-remitting MS at last count.

3) *Rational extrapolation from preclinical data to clinical trial design.* Since the EAE model is a model of the inflammatory aspect of MS, trials based on this model have targeted the clinical correlate: new lesions and clinical relapses in MS. While it is increasingly recognized that some of the chronic progressive features of MS are non-inflammatory, the field was not hindered by an effort to treat all aspects of the disease in each clinical trial.

4) *Validation of a surrogate endpoint predictive of clinical outcome*. The original beta-interferon trial was also novel in the inclusion of MRI lesion count as an outcome measure [18]. This was innovative at that time, but once its utility was demonstrated, the reliance on surrogate outcome measures has become standard, routine, and beyond question. Similar surrogate outcome measures will be necessary to develop a paradigm for a "proof of concept" trial design in AD.

LOOKING TOWARDS A MORE SUCCESSFUL FUTURE IN AD DRUG DEVELOPMENT

A "wish-list" for future directions in AD therapeutic development may be organized along the same four points listed above in reasons for success and failure:

1) *Rational use of animal models.* There may be occasional opportunities for launching clinical trials without animal data, but we are likely to continue to use animal models as a prelude to clinical trials in AD, despite their imperfections. Frustrations with animal models in other neuroscience arenas have given rise to the "STAIR" recommendations for pre-clinical evaluation of stroke therapies [19], and to the Michael J. Fox Foundation's current requirement that efficacy of any candidate therapy must be demonstrated in two distinct animal models before moving forward to clinical testing. However, neither of these strategies has

yet borne fruit, clinically speaking, so it remains to be seen whether these types of recommendations are the best path forward. Therapeutic development in AD is unlikely to be advanced by generation of more animal models or by continued debate over the relative merits of specific models or of animal models in general, keeping in mind the mantra, illustrated in the EAE-MS example, that "All models are bad, but some are useful."

Instead of revising our preclinical models, we may render animal models more useful by more careful attention to early clinical trial design. Early clinical trials should define a study population, disease stage, and dose and duration of treatment that follow logically from animal studies. Greater efforts to include "translatable biomarkers" in preclinical studies will also facilitate effective translation to clinical trials.

2) *Careful attention to study population in clinical trials.* The AD therapeutics field is attending to this issue in several important ways. There is general agreement that a clinical trial study population should be as homogeneous as possible; the challenge is finding the ideal subjects. For example, the ideal study population for clinical testing of drugs shown effective in transgenic mouse models of autosomal dominant AD would be human beings who express those genes, but the rarity of those mutations is limiting. Clinical trials in the DIAN cohort [20] and in a large Columbian kindred with autosomal dominant AD [21] are examples of how this idealized study population can be assembled, but the numbers of candidate therapies that can be evaluated with these extraordinary approaches remains quite limited.

While these carriers of well-defined highly penetrant genes for autosomal dominant AD are very rare, there may be a larger population of AD patients who, like these gene carriers, exhibit increased rates of $A\beta_{42}$ synthesis, so may be more ideal candidates for therapies that attenuate Aβ production rates. Although the vast majority of late onset AD seem to have deficits in clearance, rather than synthesis of Aβ [22], patients with early onset AD who are not gene carriers may nevertheless have increased rates of Aβ production (based on the observations with the known genes for autosomal dominant AD). It may be possible to characterize these individuals with Aβ production rates in patient-derived fibroblasts or induced pluripotent stem cells in order to identify a cohort of Aβ over-producers for a targeted clinical trial.

Some trials are also defining study populations using more common genetic risk polymorphisms such as ApoE. The ideal trial in this population would be directed at the mechanism by which ApoE promotes AD, but that trial will require a more complete understanding of ApoE pathophysiology than is currently available. In the meantime, E4 carrier status may be used to select for risk of decline in individuals with mild impairments and may also be applied in *post hoc* analyses divided by ApoE status to identify genotype-specific treatment effects (as suggested in ADCS-DHA trial).

Moving beyond genetic definition of the study population, the AD field has also moved toward a requirement for biomarker evidence of amyloid pathology as inclusion criteria for participation in anti-amyloid trials aiming at disease prevention or very early intervention, and this is expected to improve the chances of detecting treatment effects.

There are also some low-tech approaches that may be considered, starting with limiting study populations by age. For example, if older AD patients tend to have a greater degree of non-amyloid pathology underlying their clinical AD diagnosis compared to younger patients, then confining anti-amyloid trials to the younger patients who are more likely to have "pure" amyloid pathology will increase the likelihood of finding treatment effects. This raises the specter of "age-ism" and other concerns, but in light of increasing evidence that clinical AD in older patients is pathologically distinct from younger patients, matching the study population to the intervention is at least rational, and may even be essential for detecting therapeutic effects with these approaches.

Another "low tech" approach may be to require study participants to demonstrate a given level of disease activity in the year prior to study entry, along the lines of the requirements for MS trials. In the case of AD, this would mean evidence of a given rate of change over time, which would mean monitoring potential drug study candidates before randomization.

A final low-tech approach involves minimizing non-genetic heterogeneity, recognizing that environmental differences in study populations are a major confounder in the effort to identify treatment effects. This might best be achieved by efforts to promote healthy brain habits (e.g., vascular risk optimization, optimal nutrition, optimal sleep, exercise) among potential trial participants. While this would require considerable effort, imagine if the NIA-funded Alzheimer's Centers were commissioned to create a "trial-ready" cohort of subjects rather than continue another 30 years of natural history studies.

Subjects could be genotyped, biochemically phenotyped (in terms of Aβ production rates, for example), coached and optimized in consensus best practices for optimal brain health, phenotyped in terms of rate of progression, and then delivered to target-specific clinical trials.

3) *Clinical study designs that follow logically from the rationale.* Clinical trial design frequently deviates from the original rationale of the study for a variety of reasons. For example, in recognition of the fact that "ideal" subjects do not exist in sufficient numbers to fill a trial, inclusion-exclusion criteria are typically not as stringent as they should be at the time of protocol design, and they are invariably loosened further when recruitment falls behind schedule.

Further trial design compromises may be made for the sake of intellectual property. For example, in the ADCS-DHA study, part of the rationale for focusing on DHA rather than mixed omega 3 s arose from the opportunity for co-sponsorship of the study from manufacturers of a proprietary form of "pure" DHA. As described above, this departure from the epidemiological data may have reduced the potential for seeing a therapeutic effect. We describe below a "second effort" with omega 3 s which is more faithful to the epidemiology and may have a greater chance of achieving success.

4) *Development of surrogate outcome measures that predict clinical outcomes.* The ultimate validation of a surrogate outcome measure will depend on the demonstration of concomitant effects on the surrogate measure and on clinically important outcomes. In the MS world, this occurred with the original beta-interferon trial and set the course for MS drug development for the next 20-plus years. This may be achieved in AD with currently available CSF protein biomarkers, MRI measures, or PET measures, but the expense, duration of follow-up required, and insensitivity to short term change in brief proof-of-concept trials limit each of these modalities. The development of biomarkers which are more sensitive to change in the short term could greatly accelerate the pace of therapeutic development. The development of biomarkers which extend beyond Aβ and tau pathology may also facilitate development of effective interventions. We describe below three examples of alternative surrogate outcome measures being developed in studies which are currently underway.

Works-in-progress toward a paradigm for screening therapeutic strategies for AD and related disorders include:

1) The NIA-funded trial "PUFAs for the prevention of vascular cognitive impairment" (coPI's Shinto and Bowman) is an example of a single site trial of a therapeutic strategy which is strengthened by a rigorous definition of study population, an intervention that is true to the original rationale [23], and a surrogate outcome measure that reduces the numbers of subjects and duration of time needed to detect a treatment effect.

The hypothesis is that omega 3 fatty acid supplementation will reduce the progression of white matter hyperintensities in elderly subjects at risk of vascular cognitive decline, based on observations by us and others of a strong relationship between white matter hyperintensity burden and plasma omega 3 status [23]. The study population is defined by the absence of dementia, low baseline plasma levels of omega 3 s, and a requirement for a threshold level of white matter hyperintensity at baseline. The intervention is fish oil with EPA and DHA in the combination which drives the observational studies which motivated the trial, and which combines the potential neuroprotectant effects of DHA with the potential vascular benefits of EPA. The primary outcome measure is the rate of accumulation of white matter hyperintensities over three years. The trial is fully enrolled with 100 participants recruited at Oregon Health and Science University and clinical activity will be completed in 2019.

2) An NCCIH-funded clinical study of a botanical treatment for AD (PI Soumyanath) exemplifies the use of a relatively novel surrogate outcome measure for early clinical development of an AD treatment developed in an animal model. Studies in cell culture and animal models have demonstrated that *Centella asiatica* has potential as a therapeutic agent for AD because the extract attenuates mitochondrial dysfunction induced by Aβ [24–28]. In order to determine if these effects can be achieved in human subjects, a surrogate measure of brain energy production utilizing phosphorus NMR-spectroscopy will be employed in a preliminary dose-finding study. Phosphorus-NMR measurement of brain energy status has been employed productively in similar early phase clinical trials in Huntington's disease [29], but is relatively novel in AD and is well-suited to this trial based on the preclinical data. This study is at a very early stage, with IND application still under way.

3) An NCATS-funded study of CSF microRNA as biomarkers of AD (PI Saugstad) is another example of an effort to develop novel outcome measures for proof-of-concept trials in AD. Funded as part

of the extracellular RNA consortium [1], this effort involves refinement of protocols for isolating and quantifying RNA from CSF [30], and initially generated 26 candidate biomarkers from a panel of 756 miRNAs [30]. Validation of these findings in an independent sample is under way at present, and future plans include evaluation of plasma miRNA, evaluation of specificity for AD, effects of disease stage, and others. The hope is that this miRNA panel will identify new target pathways and will also serve as a rapidly responsive read-out of therapeutic effects in brief proof-of-concept clinical trials.

In conclusion, we have identified several key areas for improvement in the development of AD therapeutics, and we have initiated efforts to move the field forward. The repeated successes in other areas of clinical neuroscience prove that this is not futile, provided we learn from both the failures and successes of the past two decades.

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The End of the Beginning of the Alzheimer's Disease Nightmare: A Devil's Advocate's View

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Abstract. Although there have been so many failures in Alzheimer's disease (AD) modifying trials, there are still many compounds in the pipeline and the hope still remains that the entrance of disease-modifying treatment (DMT) for AD will positively and dramatically change the whole situation of AD treatment. However, if DMT does enter the market, it will be the beginning of a great number of challenges and problems. The current infrastructure for diagnostics of early (pre-dementia) AD does not have the capacity to meet the demands and expectations of the population. Neither is there capacity for treatment monitoring and follow-ups. If screening is considered, there will be a great risk for false positive cases and a great number of people who will have to undergo diagnostics. There will be high costs for diagnostics and treatment initially, while potential benefits will occur much later in other sectors than where the payers for treatment are. Although there are great hopes that prevention of cardiovascular risk factors and changes in lifestyle might impact the risk for dementia, there is still no consensus that this is the case. Finally, the relevance of different AD paradigms such as amyloid and tau is still a matter of discussion, particularly regarding the oldest old.

Keywords: Alzheimer's disease, costs, diagnosis, disease modifying treatment, economic simulation, predictive values, prevention, reimbursement

INTRODUCTION

Alzheimer's disease (AD) can be described as a nightmare for those affected, their families, and society. AD has devastating effects on one's life. Slowly and unavoidably, perhaps the most important part of life deteriorates—thinking, autonomy, and freedom. AD also affects one's family in many ways, including relationships, future planning, economic situation, physical care burden, and behavioral and psychological symptoms related to dementia [1].

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The societal costs are enormous. In 2015, it was estimated that the aggregated global costs of AD were about 818 billion US$ [2]. The contribution of families and friends in terms of unpaid informal care constitute about 40% of these costs. The costs of health care, community care, and long-term care put an enormous burden on any society, and the forecasts for future numbers of people with AD and other dementias is an enormous challenge for all societies.

Although there have been so many failures in AD disease-modifying trials, there are still many compounds in the pipeline and the hope still remains that the entrance of disease-modifying treatment (DMT) for AD will positively and dramatically change the whole situation of AD treatment. Furthermore, based

on epidemiological data and trends, as well as intervention trials, there are great hopes that prevention and risk reduction [3] activities also will contribute to an optimistic view—people will be diagnosed with AD or other dementias or as at risk of them much earlier than today, life for people with dementia and their families will become almost normalized, there will be huge cost savings, and the need for long-term care will be much lower than expected.

If this comes true, it is of course wonderful. However, and to paraphrase a famous statesman: The entrance of DMTs is not the end of the AD nightmare. It is not even the beginning of the end. But it is, perhaps, the end of the beginning. Why this rather killjoy view?

DIAGNOSTICS

First, and this process is already ongoing, there is the "diagnostic ribbon shift" from AD dementia to AD pre-dementia stages (such as AD-mild cognitive impairment (MCI), prodromal AD, preclinical AD, and subjective cognitive decline) [4–7].

This diagnostic ribbon shift, and perhaps combined with a strong commitment from patient advocacy organizations, results in a much larger potential target population for diagnostics. Although there is a great variability in the estimated numbers and proportions of people with pre-dementia states, the numbers that might be in need of a diagnostic process might double or perhaps triple. The difficulties in setting a pre-dementia AD diagnosis are also greater than setting an AD-dementia diagnosis because the instruments for cognitive testing face more problems with sensitivity, specificity, and predictive values in pre-dementia stages. Thus there are great expectations that biomarkers (such as cerebrospinal fluid or positron emission tomography for detection of amyloid and tau or, with a wider definition, also magnetic resonance imaging can support the diagnostic process as supplements to clinical measures [8].

Today, a great part of the biomarker-supported diagnostic process takes place at research centers and memory clinics (and similar). However, the capacity in care systems to offer a biomarker-supported diagnostic process is already limited today in high-income countries (HICs) (and in low and middle-income countries (LMICs) in reality available only for high-income groups). If a substantial increase in the demands and expectations for a pre-dementia diagnostic process will be the result of an approved DMT, the challenge for the diagnostic infrastructure will be enormous [9].

A strongly expanded biomarker-supported diagnostic process will also increase the costs (publically financed or out of pocket) for diagnostics. Even if a biomarker-supported diagnostic process will of course improve the accuracy of diagnostics, the risk for diagnostic errors (particularly false positive cases) will still remain [10]. Nice figures of sensitivity and specificity (95% for example) from memory clinics (where the prevalence of true positive cases is rather high) of any diagnostic tool might result in an extensive number of false positive cases if applied in primary care, where the prevalence is much lower. To handle this diagnostic challenge, a step-by-step process is necessary to enrich the final population for biomarker-supported diagnostics at the memory clinics. For pre-dementia AD diagnostics, primary care can, at best, identify "persons at risk" for AD dementia at a later time. Being identified as "at risk" might cause stress, particularly given the great uncertainties before a more profound and advanced diagnostic process at memory clinics (and similar) has started.

However, even at memory clinics with experienced clinicians, neuropsychologists, and biomarker-supported diagnostics, there will still be risks for diagnostic errors, particularly if an over belief in biomarkers' diagnostic capacity is the case [11]. Moreover, most people with dementia are over 75 years, and the older we get, the more we face problems in determining the reason for cognitive decline (such as AD, vascular dementia, dementia with Lewy bodies, and frontal lobe dementias) because cognitive impairment of the oldest old (such as 80 + years) has a multifactorial origin and often is part of a frailty syndrome [12].

Instead of looking at the diagnostic accuracy of single methods, the focus should be on diagnostic packages with varying contents of diagnostic tools and sequences [13]. Starting from a basic set (such as physician's exam, simple cognitive tests, and basic laboratory tests), the added value (in terms of new true positive and true negative cases) for each new added test (and the sequence of how tests are added) should be evaluated. To do everything that can be done on everyone is not realistic or justifiable (the marginal cost for the last identified case might be enormous). There is an optimum and balance in terms of cost and effectiveness in identifying true positive and negative cases and avoiding false positive and negative cases. However, cost effectiveness analyses of "diagnostic packages" hardly exist for AD. Our group has also

tried to estimate the negative effects of being falsely labeled as having (false positive) or not having (false negative) AD [14].

SCREENING?

If a DMT reaches the market, it seems reasonable to recommend screening because AD dementia has a long period without symptoms (preclinical) or with only slight symptoms (prodromal). However, screening for AD does not fulfill the necessary criteria for screening [13, 15, 16] even if there are opposing views on that [17]. In particular, the concerns about positive predictive values (PPV) are crucial. With a prevalence of about 7% in the target population and with a sensitivity and specificity of 95% (which are hardly realistic), the PPV will only be about 60%. If such a screening outcome were to be the basis for further diagnostics at memory clinics, these clinics will be flooded by cases in need of extended diagnostics. A mass screening of say, people over 65, will also need its own costly infrastructure in the community. More targeted approaches, such as in the EU-sponsored Models of Patient Engagement for Alzheimer's Disease (MOPEAD) project (http://www.mopead.eu/), might be an option, where screening questions on subjective memory problems are used as a filter. This project is ongoing, but results are so far not available.

TREATMENT MONITORING

Depending on how a potential DMT is designed, there will probably be rather complex procedures for how to make the treatment work and how to monitor it in terms of efficiency and safety. Treatment with the current symptomatic drugs can sometimes start and continue in primary care. This will not be the case with DMT. Because treatment with DMT will start early and we will likely not know how or when to terminate treatment, the numbers of patients under treatment at memory clinics will increase considerably. Because the memory clinics will also have great problems in handling the diagnostic processes (see above), the problems for memory clinics in also managing and monitoring the treatment will be extensive.

PAYERS

When the patent on donepezil expired, there was a price drop of more than 90% in a very short time in Sweden. Before that, the annual cost was about 1,500 US$ (I here use donepezil as a representative of all current drugs for AD). The lowest annual cost of donepezil today is less than 50 US$. Even if we do not know the price of a potential future DMT, it will probably be much higher than the cost for donepezil before the patent expired. Why should a stakeholder pay perhaps, say, 200 times more than for the current drugs for a new AD drug, where the within-trial (short-term) efficacy perhaps is in line with the current AD drugs and where the added clinical benefit, although statistically significant, not is so obvious? Moreover, the potential benefits will probably occur in the social care sector (long-term care) or in terms of a reduced need for informal care, while the costs for DMT will be seen in the medical sector (sometimes called perverse or imperfect incentives). Finally, there will be substantial costs for treatment in early AD (AD-MCI and mild AD dementia) for many years, where the societal costs even without a DMT are rather low. Potential benefits in terms of resource use and costs to an unknown scope will occur much later (in terms of postponing long-term care) and beyond the trial periods.

The answer is of course the label "disease modifying", implicating positive treatment effects beyond trial periods. Before empirical long-term data will be available (and hardly in terms of randomized controlled trials, but perhaps from registries?), the most common way to look at long-term effects will be health economic simulations. A model is at the same time a mathematically very complicated yet very great simplification of what might happen given a set of assumptions. I regard models as unavoidable tools, but I am also aware of the methodological problems and the skepticism around their use among many reimbursement authorities (payers, stakeholders). Simulations are highly dependent on their inputs and assumptions (empirical within-trial short-term efficacy translated to long-term effects, price of the DMT, disease progression, survival, unit costs, care system, etc.). There is also a great focus on the mathematical construction of models, and there are many simulation methods (such as Markov cohort models and microsimulations), which for many readers are hard to understand. There is also a lack of transparency in many published models. Nevertheless, models are indeed needed to get a view of the long-term effects of DMT. In a simulation paper, we evaluated the long-term effects of a hypothetical DMT with a strong positive effect (it prevented a conversion from MCI-AD to dementia AD in 50%

of the patients) [18]. To our surprise, a very effective DMT did not result in any cost savings at all. There were two reasons for this. First, people who start treatment with a DMT in MCI-AD will be treated for several years with a rather (compared to current drugs) expensive treatment while costs for care without treatment remain rather low, and second, the DMT also prolongs the survival time. In the very extensive sensitivity analysis, we varied all important and uncertain parameters, and the greatest impacts on the cost effectiveness were assumptions on survival and the price for the DMT. However, there are many potential scenarios for survival patterns (such as prolonged periods in early or late stages) as shown by Gustavsson et al., and these have implications for the cost effectiveness [19]. Under the framework of the International Pharmacoeconomic Conferences on AD (IPECAD, we are working on developing transparent open-source models (http://www.ipecad.org).

It is also important to clarify that cost effectiveness is not the same as cost savings. There is always a "societal willingness to pay" for something that is "good" and better than "usual care". Good "care" such as DMT, nursing, and psychosocial interventions costs money, and better care than current care most often implies higher costs. In health economics, this is often expressed in terms of the incremental cost effectiveness ratio, where the most common outcome is quality-adjusted life years (QALYs). However, in randomized controlled trials, it has been very difficult to show any significant effects on quality of life, both with quality of life instruments per se (dementia specific or not) and in terms of QALYs.

Most people with AD and other dementias live in LMICs [2], but all of the care concerns and discussions mainly reflect the situation in HICs. If we regard the situation in HICs as problematic, these challenges are minor compared to the situation in LMICs. If the major problem in HICs is funding and to expand an existing infrastructure, the challenge in LMICs is that the needed infrastructure for AD diagnostics and treatment is almost or completely nonexistent. Even with the current symptomatic drugs and with expired patents, the availability in LMICs is very limited [20]. To make potential DMTs available for the majority of people with AD in LMICs is thus an enormous challenge. Different pricing policies in different countries might be an option, but possibilities for parallel import and Internet commerce might threaten such solutions.

Besides the costs for the DMT, there will be a great need for investments in an extended infrastructure for diagnostics and treatment as described above. The probably rather small effects in terms of within-trial efficacy, the timing, and the "perverse incentives" aspects (see above) combined with strong competition with treatments of, for example, cancer and cardiovascular disorders present an enormous challenge. Will payers make investments in diagnostics and treatment monitoring for predementia diagnosed AD for long periods of years or even decades? It will not be easy to define "filters" to avoid a chaotic situation. Age is for ethical reasons not allowed as a discrimination factor per se, but arguments in terms of safety and weaker diagnostic accuracy in the elderly might indirectly make age a "filter".

PREVENTION?

Based on all of the problems highlighted above, why not focus on prevention? The Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) [3] has indeed changed the treatment paradigm not only for dementia, but also for AD and other similar diseases. In a simulation, we showed that a multi-domain approach, as in FINGER, can be cost effective both in terms of monetary savings and the outcome (QALYs) [21]. Another advantage is that the multi-domain approach, as in FINGER, with a focus on cardiovascular risk factors, nutrition, and lifestyle is already part of the daily work in primary care [22]. There is no great need for a new complicated and costly infrastructure. However, the working situation in primary care is often stressful with short visits (5–15 minutes), and the knowledge and interest in dementia varies considerably. In many countries, the payment system does not encourage long visits with comprehensive investigations and follow-ups (as are needed for AD and other dementias). Therefore, there is a need for a great change in how primary care works before primary care can be a strong force in dementia care in terms of diagnostics, prevention, and treatment. Furthermore, the results from FINGER need to be confirmed in similar projects. FINGER was a 2-year trial, and we do not know the long-term effects of the intervention. Also, FINGER showed a positive effect on cognition, but we do not know whether it also had an effect on the risk of conversion to dementia.

There is no contradiction between prevention and DMT. Lessons from other disease areas, such as HIV and cancer, can serve as a good framework for a wider

multidomain approach where prevention and DMT are combined [23].

THE AD PATHOGENESIS PARADIGMS

What if we suppose that the most common theories for the pathogenesis of AD are wrong [24, 25]? I am not an expert in this field, but the outcome in the struggle between "baptists" (beta amyloid protein) and "tauists" (and perhaps added with neuroinflammation) might result in a conclusion that all of them are wrong, or at least too simplistic, and thus chicken and egg discussion still remains. In my discussions about DMT above, I have assumed that at least some (One of them? Some combination?) of these paradigms are "correct" to a substantial extent, but why all the treatment failures? The multifactorial origin of cognitive impairment of the oldest old is perhaps a major reason for treatment failures in DMT trials even if biomarker-supported AD pathology is shown in the brain. An age cut-off of, say, 75 years would make the target population for DMT treatment much smaller (roughly 75% smaller), but on the other hand, the changes needed in order to obtain a positive trial outcome will probably be greater because the probability of a more "cleaner AD pathology" is greater in the "young elderly".

CONCLUSIONS

All of my concerns as "the devil's advocate" raised in this paper might label me as "anti DMT". Nothing could be more wrong. I would be very happy if a strong DMT were to be available. It is, however, necessary to be aware of the very great challenges that would remain once a DMT is on the market. To use a drastic formulation: The nightmare for a drug company will start once a drug with a DMT label for AD has shown statistically positive results in a trial!

My concerns are not unique, and most people who in different ways are involved in dementia care and research are aware of the situation, so to some extent I am "flogging a dead horse" (Swedish idiom: "battering open doors"). Nevertheless, we are waiting and longing for a "positive DMT signal". A weak "signal" (statistically significant, but clinically modest) might be nice as a support for the underlying paradigms about AD pathogenesis, and it would also be nice after so many failures, but it would hardly convince payers. A "strong" signal would probably start a cascade of demands for care from patients, families, advocate organizations, clinicians, and researchers, but the potential target populations for DMT would be so large that payers even in such a situation would still have concerns. Experiences from new and efficient treatments for hepatitis C, multiple sclerosis, and some cancers show that payers face great problems in priorities. The budget impact of a costly DMT for early AD would be enormous. Thus, it is crucial to adopt a stepwise introduction with filters, which might be problematic in relation to all of the demands for access to treatment. Such discussions are ongoing in many HICs in terms of "conditional market approval", where a treatment can be approved under specific conditions and with a demand for the producers of the drug to present follow up support for efficiency, safety, and cost effectiveness "later". Whether this is the right way forward for DMT in AD is, however, hard to say.

DISCLOSURE STATEMENT

The author's disclosure is available online (https://www.j-alz.com/manuscript-disclosures/17-9905).

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Revolution of Alzheimer Precision Neurology. Passageway of Systems Biology and Neurophysiology

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Abstract. The Precision Neurology development process implements systems theory with system biology and neurophysiology in a *parallel, bidirectional research path*: a combined hypothesis-driven investigation of systems dysfunction within distinct molecular, cellular, and large-scale neural network systems in both animal models as well as through tests for the usefulness of these candidate dynamic systems biomarkers in different diseases and subgroups at different stages of pathophysiological progression. This translational research path is paralleled by an "omics"-based, hypothesis-free, exploratory research pathway, which will collect multimodal data from progressing asymptomatic, preclinical, and clinical neurodegenerative disease (ND) populations, within the wide continuous biological and clinical spectrum of ND, applying high-throughput and high-content technologies combined with powerful computational and statistical modeling tools, aimed at identifying novel dysfunctional systems and predictive marker signatures associated with ND. The goals are to identify common biological

denominators or differentiating classifiers across the *continuum* of ND during detectable stages of pathophysiological progression, characterize systems-based intermediate endophenotypes, validate multi-modal novel diagnostic systems biomarkers, and advance clinical intervention trial designs by utilizing systems-based intermediate endophenotypes and candidate surrogate markers. Achieving these goals is key to the ultimate development of early and effective individualized treatment of ND, such as Alzheimer's disease. The Alzheimer Precision Medicine Initiative (APMI) and cohort program (APMI-CP), as well as the Paris based core of the Sorbonne University Clinical Research Group "Alzheimer Precision Medicine" (GRC-APM) were recently launched to facilitate the passageway from conventional clinical diagnostic and drug development toward breakthrough innovation based on the investigation of the comprehensive biological nature of aging individuals. The APMI movement is gaining momentum to systematically apply both systems neurophysiology and systems biology in exploratory translational neuroscience research on ND.

Keywords: Alzheimer's disease, biomarkers, integrative disease modeling, pathophysiology, precision medicine, precision neurology, systems biology, systems neurophysiology, systems pharmacology, systems theory

Abbreviations: ^{18}F-FDG-PET, ^{18}F-2-fluoro-2-deoxy-D-glucose PET; Aβ_{42}, 42-amino acid-long amyloid beta peptide; AD, Alzheimer's disease; ADD, Alzheimer's disease dementia; ADNI, Alzheimer's Disease Neuroimaging Initiative; ADO, Alzheimer's disease ontology; APMI, Alzheimer Precision Medicine Initiative; APMI-CP, Alzheimer Precision Medicine Initiative Cohort Program; APP, amyloid precursor protein; BFCS, basal forebrain cholinergic system; CSF, cerebrospinal fluid; DBS, deep brain stimulation; DLB, Dementia with Lewy bodies; DTI, diffusion tensor imaging; EEG, electroencephalography; EHRs, electronic health records; EPAD, European Prevention of Alzheimer's Dementia consortium; EPAD LCS, EPAD Longitudinal Cohort Study; EPI, echo planar imaging; FA, fractional anisotropy; fMRI, functional magnetic resonance imaging; FTD, frontotemporal dementia; ICNs, intrinsic coherent networks; IDM, integrative disease modeling; MCI, mild cognitive impairment; MD, mean diffusivity; MEG, magnetoencephalography; MMN, mismatch negativity; MRI, magnetic resonance imaging; ND, neurodegenerative diseases; NFL, nerve fiber layer; p-tau, hyperphosphorylated tau; Nold, normal elderly subjects; PDD, dementia due to Parkinson's; PET, Positron Emission Tomography; PM, Precision medicine; PMI, Precision Medicine Initiative; PoC, Proof-of-Concept; RGC, retinal ganglion cell; ROI, region of interest; rTMS, repetitive transcranial magnetic stimulation; SBML, Systems Biology Markup Language; SPECT, Single Photon Emission Computed Tomography; t-tau, total tau; tACS, transcranial alternating current stimulation; tDCS, transcranial direct current stimulation; WB-MRI, whole-body magnetic resonance imaging; WES, whole-exome sequencing; WGS, whole-genome sequencing; WM, white matter.

INTRODUCTION

A dementia syndrome is caused by a range of neurological disorders; Alzheimer's disease (AD) is the most common disease-causing dementia, accounting for 50–70% of cases. Increasing age is the most important risk factor for AD and other dementias, and as life expectancy increases and demographic aging occurs in populations around the world, the number of people with dementia is expected to continue to exponentially grow. In 2015, almost 47 million people worldwide were estimated to be affected by dementia, and the numbers are expected to reach 75 million by 2030, and 131 million by 2050, with the greatest increase expected in low-income and middle-income countries [1].

On May 29, 2017, at the 70th session of the World Health Assembly in Geneva, the World Health Organization (WHO) has unanimously adopted a global plan on dementia—the Global Plan of Action on the Public Health Response to Dementia 2017–2025—that includes targets for the advancement of dementia awareness, risk reduction, diagnosis, care and treatment, support for care partners, and research (available at https://www.alz.co.uk/news/global-plan-on-dementia-adopted-by-who).

Recent years have witnessed an increasing understanding of the molecular mechanisms related to AD. The pathogenesis of this complex polygenic neurodegenerative disease (ND) involves sequentially interacting pathophysiological cascades, including both core events, i.e., accumulation of the 42-amino acid-long amyloid-β (Aβ_{42}) peptide into amyloid plaques and self-aggregation of hyperphosphorylated tau protein to form intraneuronal neurofibrillary tangles, and downstream processes, such as generalized neuroinflammation [2, 3]. These events induce axonal degeneration [4–6] and disruption of synaptic integrity [7, 8], thus leading to synaptic dysfunction

and, ultimately, deterioration of physiological neural connectivity [9].

In spite of such advancements in understanding the disease, AD is characterized by a high degree of heterogeneity in its manifestation, progression, and response to treatment, as well as susceptibility to risk factors. Phenotypic variability is currently considered one of the biggest challenges in clinical science and clinical trial design [10]. On the one hand, the same syndrome can be caused by substantially different pathophysiological mechanisms. In order to ensure more precise and definitive AD diagnosis, biomarkers are crucially needed to detect and track disease processes in the brain. On the other hand, similar pathophysiology can present itself with distinct symptomatology across patients, suggesting that additional factors can influence disease manifestation and progression. The identity and impact of such additional factors (including genetic, epigenetic, lifestyle, and phenotypic traits) deserve further investigation. Particularly, a growing body of evidence demonstrated that a factor such as an individual's sex can modulate disease phenotype and drug response [11], thus substantially contributing to clinical heterogeneity. In AD patients, sex differences have been reported in the rate of cognitive deterioration [12, 13] and brain atrophy [14], in the absence of clear differences in amyloid or tau burden [15]. In addition, sex-genotype interaction in AD have been shown to affect both risk of onset and conversion [16] as well as response to pharmacological treatment [17, 18]. The socio-economic construct associated with the female and male position in the society (i.e., gender) can also influence disease onset and progression, as it affects education, salary, pension plans, and caregiving burden [19]. Therefore, sex and gender appear to be central drivers of phenotypic variability in AD and their role should be carefully considered when designing strategies for prevention, detection and treatment of the disease. Analysis of sex and gender effects, both alone and in combination with a variety of genetic, epigenetic, and phenotypic traits, should be the first step toward a more personalized and patient-centered approach to AD.

THE PRECISION NEUROLOGY PARADIGM IN ALZHEIMER'S DISEASE

Breakthrough conceptual shifts have recently commenced to emerge in the field of AD and other ND, highlighting the presence of risk and protection factors and the non-linear dynamic continuum of complex pathophysiologies along a wide spectrum of multi-factorial brain proteinopathies. Substantial advancements in detecting, treating, and preventing AD are expected to evolve through the generation and the systematic implementation of a strategy based on the precision medicine (PM) paradigm [20, 21], whose establishment requires the implementation of an array of integrated disciplines and technological developments such as the "omics" approaches, neuroimaging modalities, cognitive assessment tests, and clinical characteristics. These converge to several domains that need to be analyzed according to the systems theory paradigm [22]. This allows for the conceptualization of novel and original models to elucidate all systems levels, assessed by systems biology and systems neurophysiology (Fig. 1), and the different types of spatiotemporal data characterizing the genetically, biologically, pathologically, and clinically heterogeneous construct of "AD" [21]. Thus, systems biology and systems neurophysiology permit to delineate the multivariate and combinatorial profiles of genetic, biological, pathophysiological, and clinical markers reflecting the heterogeneity of this condition. Thanks to fundamental advances in research technology, we got new and better performing analysis tools to register and create comprehensive brains maps and record dynamic patterns across different systems: from molecules, neurons to brain areas. Particularly, systems neurophysiology will aim at showing how computational network models can elucidate the relationship between structure and dynamic function in brain networks, as demonstrated by recent findings in time-dependent functional connectivity measured with non-invasive neuroimaging techniques.

The transition to PM from the traditional model does not occur overnight. But the more we build innovative and interdisciplinary networks with partners, the faster and more effectively we can see the changes happening. To fulfill on the promise of PM, there needs to be a new ecosystem with partnerships of multiple stakeholders who collaborate to find creative and novel solutions. Such a new ecosystem, comprised of academic and community providers, industry, professional societies, government, consumers, and patient advocacy groups, could advance the following pilot initiatives on a local, national and potentially international scale.

In order to advance the development of the PM paradigm in AD, the international Alzheimer PM Initiative (APMI) and its planned Cohort Program (APMI-CP) (Fig. 2) have been recently launched by our consortium and thematically linked to the U.S. Precision Medicine Initiative (PMI) (available

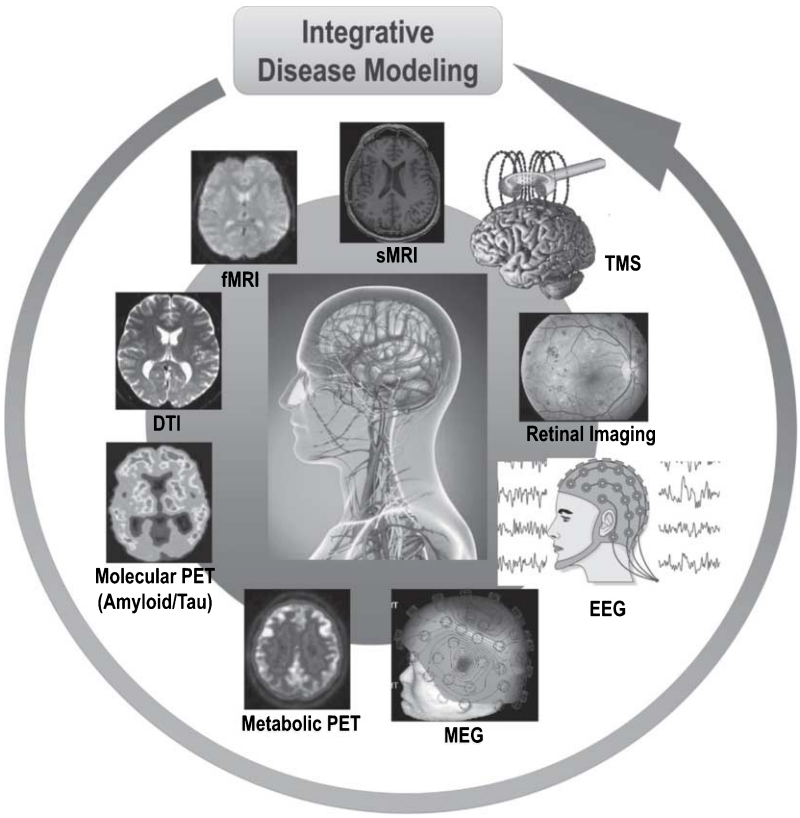


Fig. 1. Cohorts stratified according to different neuroimaging modalities and methods are integrated in the disease modeling for classification and prediction of subsets of AD and other ND patients. The paradigm of systems neurophysiology aims at studying the fundamental principles of integrated neural systems functioning by integrating and analyzing neural information recorded in multimodal fashion through computational modeling and combining data-mining methods. This paradigm may be used to decode the information contained in experimentally-recorded neural activity using analysis methods that are able to integrate the recordings of simultaneous, single-modality brain cell activity such as fMRI or EEG to generate synergistic insight and possibly infer hidden neurophysiological variables. The ultimate goal of systems neurophysiology is to clarify how signals are represented within neocortical networks and the specific roles played by the multitude of different neuronal components. AD, Alzheimer's disease; DTI, diffusion tensor imaging; EEG, electroencephalography; MEG, magnetoencephalography; fMRI, functional magnetic resonance imaging, sMRI, structural magnetic resonance imaging; ND, neurodegenerative diseases; PET, positron emission tomography; TMS, transcranial magnetic stimulation

at https://www.whitehouse.gov/precision-medicine) and the U.S. "All of Us Research Program", evolved from the U.S. PMI Cohort Program (available at https://www.nih.gov/research-training/allofus-research-program) (Table 1). Four pioneering translational neuroscience research programs—"MIDAS", "PHOENIX", "POSEIDON", and "VISION"—have been developed and launched in an interdisciplinary local network by our group at the APMI and APMI-CP initiation site Paris, France, at the Sorbonne University (*Sorbonne Université*) and at the Pitié-Salpêtrière University Hospital, Institute for Memory and Alzheimer's Disease (*Institut de la Mémoire et de la Maladie d'Alzheimer*, IM2A) and the Brain and Spine Institute (*Institut du Cerveau et de la Moelle Épinière*, ICM) in Paris to organize, combine, and integrate the components of systems biology and neurophysiology in order to facilitate the development of PM in AD, a model approach for other proteinopathies/ND of the brain. In this regard, following the APMI conceptual framework, mono-center pilot APMI subcohorts spanning from early asymptomatic preclinical populations to prodromal to dementia late stage populations, namely *INSIGHT-preAD, Predict-MA PHRC, RESPIR*, and *SOCRATES*, have been established at our central clinical recruitment site, the IM2A. These pilot APMI cohorts allow for the standardized academic university-based expert center inclusion of both cognitively intact individuals at risk for AD and patients with a full range of ND and provide an assortment of unique heterogeneous and multidimensional data. The research using these pilot AMPI cohorts is performed under the structural framework of the

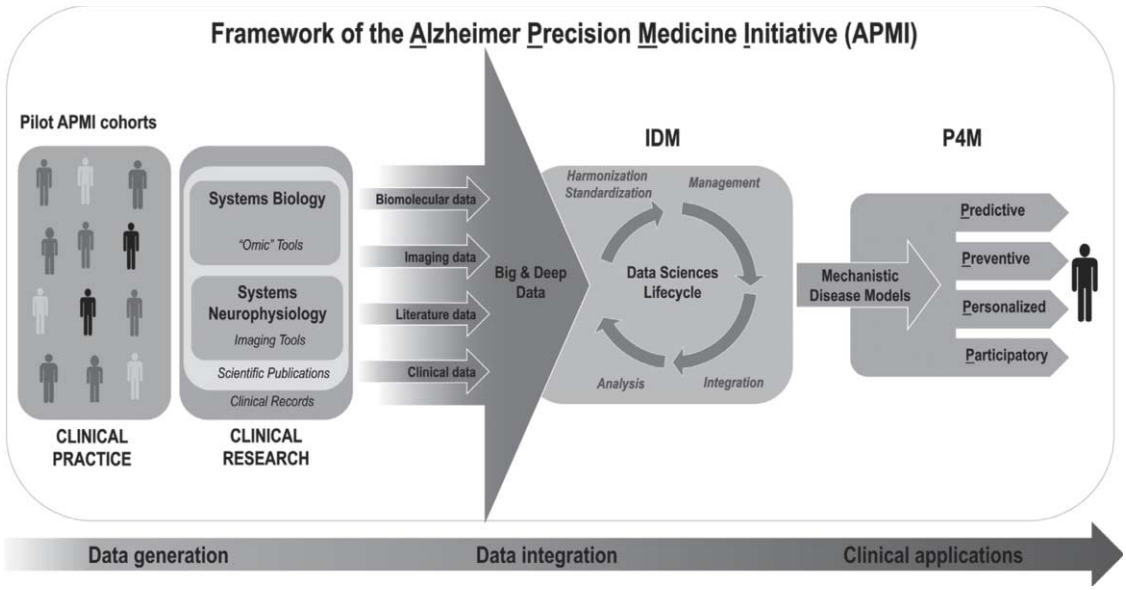


Fig. 2. Translational bench-to-bedside data flow within the conceptual framework of the Alzheimer Precision Medicine Initiative (APMI). The IDM-based "Data Sciences Lifecycle" takes advantage of both data-driven and knowledge-driven approaches so that both quantitative data (biomolecular, neuroimaging/neurophysiological, and clinical data) and qualitative data (collected from scientific literature and on-line media)—generated through the application of systems biology and systems neurophysiology paradigms—are represented in a harmonized, standardized format to be prepared for proper management within an integrative computational infrastructure. Indeed, the resulting heterogeneous, multidimensional big and deep data are harmonized, standardized, and integrated via computational and data science methods in the form of mechanistic disease models, according to the IDM conception. Disease-specific integrative computational models play a key role in the IDM paradigm and represent the foundations for "actionable" P4M measures in the area of AD and other ND. As a result, the integrative disease models are anticipated to support decision making for: 1) early diagnosis of brain disease progression with mechanistic biomarkers (predictive), 2) screening populations and stratifying individuals at high risk of developing ND based on mechanistic co-morbidities in order to reduce the likelihood of disease and disability (preventive), 3) tailoring treatment to the right patient population at the right time (personalized), and 4) optimizing "actionable" plans for the benefit of patients based on patient-oriented information gathered in EHRs and on patients' feedback reported in social media. Internet has greatly enabled the participation of individual patients in the healthcare through sharing their experiences in various social media and other online resources (participatory). The output is anticipated to be an "actionable" model that permits the prediction of the trajectory of individual patient-centric detection or treatment within the implementation of the P4M paradigm. APMI, Alzheimer Precision Medicine Initiative; EHRs, electronic health records; IDM, integrative disease modeling; ND, neurodegenerative diseases; P4M, Predictive, Preventive, Personalized, Participatory Medicine. Modified from [21].

newly established Sorbonne University – "Clinical Research Group in Alzheimer Precision Medicine" (*GRC n° 21), Sorbonne Universit*é – "*Groupe de Recherche Clinique – Alzheimer Precision Medicine*") (GRC-APM). The major objective of the Sorbonne Université GRC-APM is to accelerate the reformation of traditional Neurology, Psychiatry, and Neuroscience embracing the PM paradigm, based on complex systems theory, using systems biology and systems neurophysiology, big data science, and biomarker-guided integrative disease modeling (IDM) to improve detection, classification, and therapy development in AD and other ND.

The implementation of PM in AD is expected to result into a novel, original scientific taxonomy and a distinguished working lexicon and terminology (see Table 2) for reality-based medicine, which detects evidence from real-life scenarios.

An appropriately integrative understanding of AD will be propelled by advances in molecular technology and data processing that will allow generating, analyzing, interpreting, and storing huge amounts of heterogeneous and multidimensional data, termed big data. Big data in AD can be used to improve our current mechanistic understanding of the disease through the application of different computational and data science methods, under the theoretical framework of IDM [23]. Multimodal big data integration is essential to understand the link between elements from large-scale neurobiological systems such as protein interaction and genetic regulatory networks, synaptic connections and anatomical projections among brain areas. Usually, these data come from multiple levels of organizations or involve different domains of biology and data types (Fig. 3).

Table 1
The five pillars of the Alzheimer Precision Medicine Initiative (APMI). The mission of APMI is to transform Neurology and Neuroscience embracing Precision Medicine (or Precision Neurology) based on complex systems theory using integrative disease modeling (IDM) to facilitate health care solutions for brain proteinopathies, protein misfolding disorders, and neurodegenerative diseases, such as Alzheimer's disease (AD). This is facilitated through five breakthrough theoretical scientific advances, as follows:

Concept	Comment
(1) The emergence of the **"precision medicine" paradigm**	Discovery and development of treatments targeted to the needs of individuals on the basis of *systems biology technology* using genomic biomarker, phenotypic, or psychosocial characteristics that distinguish a given individual from others. Inherent in this definition is the goal of impacting pathophysiological progression at early disease stages and clinical outcomes at later stages and minimizing unnecessary side effects for those less likely to have a response to a particular treatment supported by pharmacogenomics. The *convergence of genetics/genomics/transcriptomics, bioinformatics, neurodynamics, neuroimaging, and connectomics along with other technologies such as cell sorting, epigenetics, proteomics, lipidomics and metabolomics,* is rapidly expanding the scope of precision medicine by refining the staging and classification of disease, often with important prognostic and treatment implications. Among these new technologies, genetics and next-generation DNA sequencing methods are having the greatest effect.
(2) The emergence of the **"systems biology" paradigm**	Systems biology represents an integrated and deeper investigation of interacting biomolecules within cells or organisms. This approach has only recently become feasible as *high-throughput technologies* including cDNA microarrays, mass spectrometric analyses of proteins and lipids together with rigorous bioinformatics have evolved. High-content data point to convergent pathways among diseases, which transcend descriptive studies to reach a more integrated understanding of neurodegenerative disease pathogenesis and, in some instances, highlighting 'druggable' network nodes.
(3) The emergence of the **"systems neurophysiology and complex network" paradigm**	This is due in large part to advances in mathematics, computer science and statistical methods applied to neuroimaging and neurophysiology; instead of thinking of the brain as a set of modules (i.e., individual brain regions) that perform specific cognitive functions, the network paradigm argues that cognitive functions are performed by dynamic interactions among different brain areas, i.e., by *dynamically formed complex structural and functional networks* of brain regions.
(4) the emergence of **"neural modeling" paradigm**	This paradigm is required by the *complex network paradigm*, since, in order to deal with the large complexity of the dynamic interactions among multiple brain regions, one must employ advanced mathematical and computational methods.
(5) The emergence of **"integrative disease modeling" (IDM) paradigm**	This is an evolving knowledge-based paradigm in translational research that exploits the power of advanced computational methods to collect, store, integrate, model, and interpret accumulated disease information across different biological scales, i.e., from molecules to phenotypes. **IDM** is a new paradigm at the core of translational research, which prepares the ground for transitioning from descriptive to mechanistic representation of disease processes. Given the tremendous potential of **IDM** in supporting translation of biomarker and drug research into clinically applicable diagnostic, preventive, prognostic, and therapeutic strategies, it is anticipated that *computer-readable disease models* will be an indispensable part of future efforts in the **P4 medicine** research area.

To be effective, PM needs to exploit advanced tools for collecting/managing/examining big data. Particularly, thanks to outstanding progresses in information technology, the development and implementation of electronic health records (EHRs) enable gathering/preserving longitudinal health-care records and clinical data at highly limited costs. Furthermore, the adoption of personal mobile technologies, namely phones, apps, wearables, in-home devices, as innovative ways to collect health information (mobile health or "m-health") is becoming a common practice. These devices allow the accumulation of clinically relevant information in a more ecological/natural environment and the improvement of patient care. High-volume and dense data generated from progressively more sophisticated software applications can enrich self-reported information on both lifestyle and environment, thus providing researchers with a well-defined vision of these factors, previously difficult to obtain.

Being rooted in a multidimensional data-driven approach, PM is expected to upgrade the prevention and treatment of AD to a higher level of individualization, promoting a shift toward every single preclinical participant at risk rather than late stage patients and disease in general. This goal will be achieved mainly through the identification and validation of reliable biomarkers, which will allow better classifying patients by their probable disease risk, prognosis, and/or response to preventive measures and treatment [20, 21]. To date, PM (in general) and biomarker-guided therapeutic strategies (in particular) have witnessed their broadest applications in the field of oncology. The Food and Drug Administration (FDA) has recently approved for the first time a cancer treatment based on the presence of specific molecular aberrations rather than on the tumor's anatomical origin. Pembrolizumab (a humanized antibody used in cancer immunotherapy) has been

Table 2
Evolving lexicon and terminology within the Alzheimer Precision Medicine Initiative (APMI) framework

Concept	Abbreviation	Definition
Big Data		A repository of large amounts of data sets generated by data mining tools. Big Data includes information obtained through systems theory- and, knowledge-based approaches and clinical records.
Biomarkers	BMs	A defined characteristic that is measured as an indicator of normal biological processes, pathogenic process, or response to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiological characteristics are types of biomarkers. A biomarker is not an assessment of how an individual feels, functions, or survives. Categories of biomarkers include: susceptibility/risk biomarker, diagnostic biomarker, monitoring biomarker, prognostic biomarker, predictive biomarker, pharmacodynamics/response biomarker and safety biomarker.
Data Science		Interdisciplinary field about processes and systems to extract knowledge from data in different forms – either structured or unstructured – which is a continuation of some of the data analysis fields including statistics, artificial intelligence, machine learning, data mining, and predictive analytics.
e-Health		Term indicating healthcare practice supported by electronic processes and communication. It can also include health applications and links on mobile phones, referred to as **mobile health (“m-health**”: smart personal mobile devices, such as phones, wearables, in-home devices and Apps, collecting health information aimed at improving patient care). The term can also encompass a range of services or systems that are at the edge of medicine/healthcare and information technology, including: electronic health records (**EHRs**). These indicate a systematized gathering of population electronically-stored health information and clinical data in a digital format. These registries can be shared across different health care settings through network systems.
European Prevention of Alzheimer’s Dementia Consortium	EPAD	Pan-European initiative whose objective is to establish a shared platform to design and conduct phase II Proof-of-Concept (PoC) clinical trials specifically aimed at developing novel treatments for the secondary prevention of AD.
Genomic Medicine		Discipline utilizing personal genomic information (see also the definition of “Personal Genomics”) for diagnostic characterization and the development of therapeutic plans.
Integrative Disease Modeling	IDM	Multidisciplinary approach to standardize, manage, integrate, and interpret multiple sources of structured and unstructured quantitative and qualitative data across biological scales using computational models that assist decision making for translation of patient-specific molecular mechanisms into tailored clinical applications.
“Omics” or “Omic” disciplines		High-throughput screening tools aimed at fully collecting, characterizing and quantifying pools of biological molecules (DNA sequences, transcripts, miRNAs, proteins/peptides, metabolites/lipids) that translate into the structure, function, and dynamics of an organism and/or whole organisms.
“One-size-fits-all” approach		Traditional approach used for the development of early detection, intervention, and prevention options, where biomarker candidates are being validated against the plethora of heterogeneous clinical operationalized syndromes, rather than against genetically (risk profile) and biologically (i.e., based on molecular mechanisms and cellular pathways) determined entities.
Ontology		Formal naming and designation of the types, properties, and interactions of the entities that really or fundamentally exist for a specific domain of discourse.
P4 (Predictive, Preventive, Personalized, and Participatory) Medicine	P4M	Translational medicine component of the Precision Medicine paradigm. It is a clinical practice model aimed at applying knowledge, tools, and strategies of systems medicine. It involves generation, mining, and integration of enormous amounts of data on individual patients to produce predictive and “actionable” models of wellness and disease.
Personal Genomics		Branch of genomics that provides support in predicting the likelihood that an individual will be affected by a disease. It helps personalize drug selection and treatment delivery to get the best care, thus playing a crucial role both in predictive and personalized medicine, according to the PM paradigm.
Personalized Medicine		Component of the P4M aiming at tailoring treatment for individual patients in contrast with “one-size-fits-all” or traditional “magic bullet drug” approach.

(*Continued*)

Table 2
(*Continued*)

Concept	Abbreviation	Definition
Precision Medicine	PM	Translational science paradigm related to both health and disease. PM is a biomarker-guided medicine on systems-levels taking into account methodological advancements and discoveries of the comprehensive pathophysiological profiles of complex polygenic, multi-factorial neurodegenerative diseases (proteinopathies of the brain). It aims at optimizing the effectiveness of disease prevention and therapy, by considering (customized) an individual's specific "biological make-up" (e.g., genetic, biochemical, phenotypic, lifestyle, and psychosocial characteristics) for targeted interventions through P4M implementation.
Systems Biology	SB	Evolving hypothesis-free, exploratory, holistic (non-reductionistic), global, integrative, and interdisciplinary paradigm using advances in multimodal high-throughput technological platforms that enable the examination of networks of biological pathways where elevated amounts of structurally and functionally different molecules are simultaneously explored over time at a system level (i.e., at the level of cells, group of cells, tissues, organs, apparatuses, or even whole organisms).
Systems Medicine	SM	Holistic paradigm applying systems biology-based strategies to medical research. It aims at integrating a variety of considerable biomedical data at all levels of the cellular organization (by employing global, integrative, and statistical/mathematical/computational modeling) to explicate the pathophysiological mechanisms, prognosis, diagnosis, and treatment of diseases.
Systems Neurophysiology	SN	Paradigm aimed at studying the fundamental principles of integrated neural systems functioning by integrating and analyzing neural information recorded in multimodal fashion through computational modeling and combining data-mining methods. This paradigm may be used to decode the information contained in experimentally-recorded neural activity using analysis methods that are able to integrate the recordings of simultaneous, single-modality brain cell activity such as functional magnetic resonance imaging or electroencephalography to generate synergistic insight and possibly infer hidden neurophysiological variables. The ultimate goal of systems neurophysiology is to clarify how signals are represented within neocortical networks and the specific roles played by the multitude of different neuronal components.
Systems Pharmacology	SP	Science of advancing knowledge about drug action at the molecular, cellular, tissue, organ, organism, and population levels" (http://www.aaps.org/Systems_Pharmacology/).
Systems Theory	ST	Translational research theory of the Precision Medicine paradigm. It is an interdisciplinary conceptual framework allowing for the conceptualization of novel/original models to extract and explicate all systems levels and different spatiotemporal data types of complex polygenic diseases.

Modified from [21].

granted approval for adult and pediatric patients with metastatic or unresectable, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumors [24]. The implementation of PM in ND currently impels researchers to envision a cross-trans-fertilization from such more advanced fields of medicine. In this setting, the repurposing of some previously approved mechanistic anticancer drugs for ND may offer the potential to reduce both the cost and time to achieve licensed approval status. For instance, tyrosine kinase inhibitors like bosutinib [25] and masitinib [26] (which represent a standard approach for anticancer treatment) have shown promising clinical results in patients with amyotrophic lateral sclerosis and can also exert neuroprotective actions in other ND through the activation of autophagy. The search basin for anticancer drugs repositionable for neurodegeneration will ultimately require data-driven approaches grounded on specific biomarker data; such a strategy is aimed at identifying pathophysiological commonalities, potentially common molecular alterations between cancer and ND [26].

Apart from treatment, another important aim of PM in AD will be the preclinical detection of pathophysiology at its earliest stage and related early disease initiation and the implementation of preventive interventions at the individual level. This goal may be achieved through an integrated analysis of genetic, biomarker, imaging, and clinical characteristics that distinguish one individual from others. To achieve this goal, the availability of reliable multimodal biological indicators—biomarkers—will be required [27–34]. In this regard, several potential biological markers have been identified

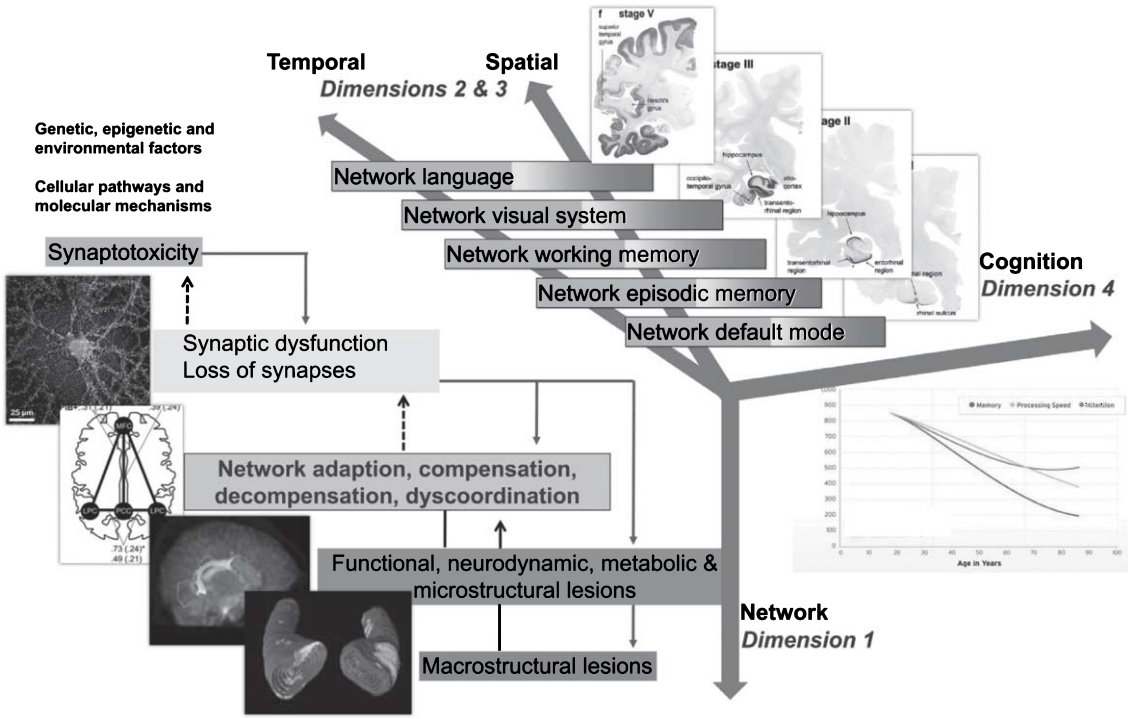


Fig. 3. Model of non-linear dynamic temporo-spatial progression of neural network disintegration and complex brain systems failure in relation to pathophysiology of AD. Four dimensions of pathophysiological processes in AD. Dimension 1 occurs at the level of neuronal networks (coded green to red). Dimension 1 can begin extremely early in form of synaptic dysfunction and/or synaptotoxic molecular agents, thus altering the balance of the neuronal network. Dimension 2 & 3 can be regarded as the temporal and spatial spreading from almost exclusively default mode to episodic memory networks to temporal, parietal and frontal neocortical associative areas responsible for working memory, language and/or visual processes. Every one of these complex systems can experience a variable degree of decompensation (see Dimension 1), from adaptation to compensation to massive decompensation and widespread disorganization. Dimension 4 is essentially the integration of Dimensions 1 and 2 and 3 into late-stage clinically symptomatic and syndromatic cognitive and later behavioral and psychopathological dysfunction and decline. It is therefore clear how this complex, multi-scale and multilayer association of networks can be partially robust to "insults" if sufficient compensatory mechanisms are in place, but also extremely and randomly fragile if adaptation and compensation fails at any level. Sufficient decompensation in Dimension 1 will turn into a malfunction in Dimension 2 and 3 and, in turn, substantial decompensation in Dimension 2 and 3 will turn into malfunction in Dimension 4 (i.e., mild cognitive impairment, clinical dementia syndrome). AD, Alzheimer's disease.

across the full spectrum of AD, from preclinical to prodromal to clinical stages [35–41]. This includes different categories, as follows: 1) neurogenetics/neuroepigenetics markers [42–45]; 2) neurochemistry markers [4, 46–48], including both cerebrospinal fluid (CSF) [49–55] and blood (plasma/serum) markers [56–63]; 3) markers derived from structural/functional/metabolic neuroimaging [64–68]; and 4) neurophysiology/neurodynamic markers [69]. Moreover, opinions of regulatory agencies and industry stakeholders in AD biomarker discovery area are regularly in discussion and development [70, 71]. The integration and recomposition of the experimental information obtained from biomarker studies through the systems biology and systems neurophysiology paradigms will ultimately allow to improve patient care and clinical outcomes through the PM paradigm [72] in line with the Institute of Medicine (IOM) Committee Recommendations for Advancing Appropriate Use of Biomarker Tests (companion diagnostics) for Molecularly Targeted Therapies [73].

Starting from these premises, PM can be conceptualized as a biomarker-guided medicine. According to FDA and the NIH Biomarkers, Endpoints, and other Tools (BEST) Resource, biomarker categories can be categorized as follows: 1) susceptibility/risk biomarker, 2) diagnostic biomarker, 3) monitoring biomarker, 4) prognostic biomarker, 5) predictive biomarker, 6) pharmacodynamic/response biomarker, and 7) safety biomarker [74]. Unfortunately, any attempt to provide such a clear-cut classification in the AD field remains problematic. For example, "amyloid positivity" is widely considered both a diagnostic and predictive biomarker; however, this may not be the case at an individual

level [74]. To target "individual variability" will ultimately require analyzing multiple biological pathways inexpensively, quickly, and sensitively. The increasing adoption of next generation sequencing in clinical practice has been recently driven by reducing costs and high-throughput analytical methods. In this setting, unbiased whole-genome sequencing (WGS) and whole-exome sequencing (WES) represent major milestones in the area of genomic medicine since they allow the complete elucidation of the genomic determinants of a specific AD patient's heritable make-up, and thus are among the most comprehensive tools for future clinical applications [74, 75]. Moreover, upcoming commercially available genetic tests, e.g., gene-based assays, implementing polygenic risk scoring for assessing AD onset risk, are currently in late stage clinical development. In particular, a 90% maximum prediction accuracy via polygenic risk scoring can be accomplished by predictors of genetic risk based on genomic profiles [76]. It is generally acknowledged that an individual's health, response to environmental and lifestyle factors, susceptibility to pathophysiology/syndromes/diseases, and tolerability/response to treatments are indeed impacted to a varying degree by their own unique biological (genetic/genomic/molecular) profile. Thanks to progress in the area of personal genomics, it is possible to identify the genetic/genomic predisposition of an individual for some common diseases, carrier status for inherited diseases, and adverse reactions to common drugs. Personal genomics provides support in predicting the likelihood that an individual will be affected by a disease and may help personalize drug selection and treatment delivery to get the best possible care, thus playing a key role in predictive and personalized medicine, in the framework of the PM paradigm [77]. In this regard, the 23andMe Personal Genome Service (PGS) Test (available at https://www.23andme.com/en-gb/) uses a qualitative *in vitro* molecular diagnostic system used for detecting variants in genomic DNA isolated from human adults specimens (saliva) that will provide information, i.e., delivering and interpreting genetic health risk (GHR) reports, to users about their genetic risk of developing a disease to inform lifestyle choices and/or conversations with a healthcare professional. Specifically, GHR reports have already been authorized by the FDA for late-onset AD and Parkinson's disease and the following diseases: hereditary thrombophilia, alpha-1 antitrypsin deficiency, Gaucher disease, Factor XI deficiency, celiac disease, G6PD deficiency, hereditary hemochromatosis, and early-onset primary dystonia (available at https://www.accessdata.fda.gov/cdrh_docs/pdf16/DEN160026.pdf). Based on the gene expression profiles generated by GenomeDx Biosciences Decipher Genomics Resource Information Database (Decipher GRID®), a recent analysis showed that the genomic signature PAM50, normally applied to breast cancer patients to determine their risk of reappearance, can be used in prostate cancer as well for predicting which individual may take advantage from early initiation of post-operative androgen deprivation therapy, thus delivering a potential clinical tool to customize the treatment of prostate cancer. This personalized selection of patients will ameliorate treatment outcomes and prevent many patients from unnecessary risks of toxicity [78].

Differently from the invariable genetic/genomic information, an individual's proteomics/peptidomics and metabolomics/lipidomics profile may be modified and vary over time. Figure 4 provides an up-to-date summary of currently available "omics" technologies (genomics, transcriptomics, miRNomics, proteomics, metabolomics) and how they can be used to disentangle different systems biomarker categories [79]. At present, the majority of the documented candidate biomarkers originate from genomic and proteomic disciplines. This might be due to the higher stability of the signal and standardization achieved by using genomic and proteomic tools compared to other available "omic" methodologies. In addition, the better stability of proteins *versus* mRNAs might account for the greater availability and progress in discovery and validation of proteomic markers compared to, e.g., transcriptomic approaches [79]. The appropriate interpretation of the obtained high-throughput data in the context of the disease molecular pathophysiology and its specific treatment is considered the rate-limiting step in the biomarker discovery and validation process. As a result, "omics" data sets need to be rigorously identified, extracted, and interpreted in order to deliver valuable biological information [79].

Within the PM framework, it has been proposed to screen and detect unsuspected age-related neurodegenerative diseases as early as possible in cognitively healthy potentially preclinical affected adults. As far as AD is concerned, it has been hypothesized that such a screening program—based on WGS combined with whole-body magnetic resonance imaging (WB-MRI), metabolomics screening, constant heart monitoring, pedigree analysis, microbiome sequencing, and standard laboratory tests—could identify people at risk of developing clinical AD decades in

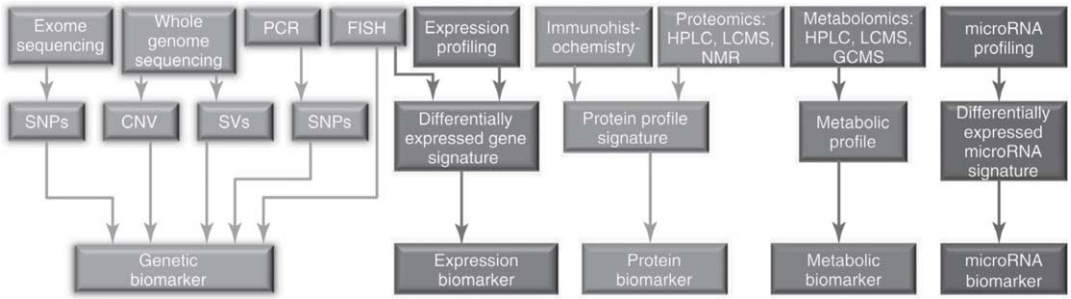


Fig. 4. Overview of the currently available technologies and the resulting biological marker categories used for biomarker discovery in preclinical and clinical research. CNV, copy number variations; FISH, fluorescence in situ hybridization; GCMS, gas chromatography mass spectrometry; HPLC, high-performance liquid chromatography; LCMS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; SNPs, single nucleotide polymorphisms; SVs, structural variations. Reproduced with permission from [79].

advance. Controversies still exist, however, regarding both the high costs inherent to this approach and the potential risks of false-positive results and overdiagnosis [80].

Very recently, a pilot study has been conducted to investigate the impact of WGS in healthy subjects examined within a primary care context. Although several potentially pathogenetic variants were identified, only a fraction of the carriers demonstrated overt clinical signs or symptoms, indicating that the expected clinical phenotype would develop later during progression of pathophysiology. Although integrating genome sequencing and other sequencing methods into the day-to-day practice will undoubtedly provide unprecedented preventive opportunities, a careful sample size determination will be necessary for achieving a sufficient statistical power to detect a clinically meaningful effect size [81].

To aid PM fully coming to life in the field of ND, the interplay of "omics"-based techniques and sequencing methods is paramount, since the availability and increasing standardization of high-throughput big data will, through adequate IDM supported by advances in data science, allow creating new biomarker-guided targeted preventive and therapeutic opportunities [20, 21]. Therefore, the use of advanced sequencing methods and of "omics"-based screening of pathophysiological disease states is anticipated to result in enhanced personalized and precise, both preventive and therapeutic, interventions by disclosing accurate patterns of pathophysiological biomarkers and molecular signatures underlying the biological mechanisms progressing non-linear dynamic in specific disease states in individual patients [82]. Extensive efforts are presently performed to explicate gene-protein links, key molecular pathways functions, protein-protein and signaling network organization, and organism-level responses *via* high-throughput biological data at different time points (e.g., global gene expression and comprehensive proteomic data) [83].

In this context, it is important to note that, so far, a major obstacle to our understanding and to the development of possibly novel stratification approaches for AD is, as mentioned, the fragmentation of previous research (single-center, single-method studies). Neuroscience has been highly productive, but its progress can also be somewhat unsystematic and remote to clinical practice. That said, so far conventional "big data" analytics techniques have failed to provide the qualitative change which is indispensable to provide a mechanistic (and not only statistical) understanding of AD pathophysiology, which in turn is instrumental to formulating personalized treatment strategies. A first step, as mentioned above, is the integration of complex and high-dimensional information from hundreds or thousands of patients contained in "big data" repositories. However, this alone is not sufficient; "big data" need to be turned into "smart data" by injecting not only novel methodologies but also expert knowledge and targeted clinical hypotheses. This poses a major analytics challenge, as neither single national-level studies nor single biomedical or technical disciplines can tackle the problem on their own. A number of potentially disease-modifying clinical development programs in AD have failed so far [84], and in addition we are in serious need of novel out-of-the box preclinical models that can generate actionable knowledge, either in research or, eventually, therapy. This is why, while computational and statistical modeling are increasingly invaluable

in AD research, it is necessary to go beyond purely descriptive data-analysis techniques (e.g., techniques that identify associations between certain data and phenotypes). Additional efforts are needed to inject specific domain competencies which can be formalized mathematically into predictive models that can disclose how specific components of pathogenic pathways interact within complex brain networks, across molecular to cellular and systems scales. Such predictive models should, as far as possible, include realistic representations of neurobiological processes and mechanisms that allow direct comparison to experimental settings and, ultimately, pave the way to discover new strategies for targeted control and intervention. In this respect, it is also essential to form additional private-public partnerships with a strong focus on data sharing and pathway-based analysis. With this type of integrative approach, successful real-world examples of advanced simulation have already generated tangible support for clinical trials in AD.

SYSTEMS BIOLOGY OF ALZHEIMER'S DISEASE

The polygenic multifactorial nature of AD and other complex proteinopathies of the brain with progression to ND is widely recognized. Although several mechanisms have been identified that may have a role in the pathogenesis of AD and other ND, the molecular and temporal dynamics of the biological processes that lead to onset and progression of diseases such as AD remain to be well-understood on a system level. Complex chronic diseases such as AD are thought to result from an interplay between environmental, genetic, and epigenetic factors. State-of-the-art "omics" techniques such as genomics, epigenomics, transcriptomics, proteomics, and metabolomics offer remarkable promise as research tools to decipher the dynamics and biological nature of the pathogenesis ultimately leading to neurodegeneration and a spectrum of clinical neurological phenotypes for which predictive markers and selective therapeutic tools are needed. Breakthrough advances in genetic and genomic technologies are making global genome sequencing possible, affordable, and clinically practical through advanced NGS technologies. New genetic technologies, however, provide a crucial basis to the understanding of the complex pathophysiological pathways involved in proteinopathies/ND.

The concept of complex multiscale systems (consisting of macromolecules that reciprocally interact with each other in dynamic modular complexes and networks) as the underlying foundations of life has been first proposed more than 50 years ago [85]. Over the past decades, we have gained detailed insights into the structure, regulation, and function of different molecular and cellular systems, which are currently viewed as building blocks or inventories of working parts. However, the main challenge ahead is to clarify how these single agents are reciprocally associated by multiple interactions across distinct system levels and networks of structural and functional organization (e.g., DNA-protein, RNA-protein, protein-protein, protein-metabolite networks, interactomics). Major challenges exist for the development of reliable holistic models that are based on unbiased data-integration workflows and that could highlight the properties of complex biological structures, for which the whole is often greater than the sum of their parts. In this context, the main goals of systems biology in the field of ND research are as follows: 1) to characterize complex systems and/or networks in a straightforward, viable manner, by probing key layers of molecular regulation and expression on a genome-wide level and 2) to integrate different genome-wide data sets in a multidimensional manner—that is, across different layers of molecular regulation, timescales, cell types and so on—in order to generate comprehensive *in silico* models of ND that show the best balance between coverage and selectivity, reduce model space down to manageable numbers of highly-prioritized testable hypotheses, and are biologically precise. This will shed more light on how complex diseases may be conceptualized as a result of altered networks states [86] caused by multifactorial perturbations, which is expected to foster marker and target discovery. Under this theoretical framework, the dynamics and biology of ND processes scrutinized by systems modeling and systems biology can be more comprehensively understood. This may be achieved via a two-step approach consisting of initial animal studies followed by confirmation and validation in clinical cohort programs [87] or via an approach consisting of molecular and clinical studies in cohorts, for example the search for predictive marker signatures, followed by studies in experimental models of ND of biological and therapeutic significance associated with such marker signatures. Numerous disease conditions in humans (including proteinopathies/ND, cardiovascular disorders, malignancies, the metabolic syndrome, and diabetes) have

a highly complex biological nature that cannot be entirely and adequately captured through the investigation of single linear molecular alterations. Besides being multifactorial, such diseases are primarily caused by altered essential networks required for the correct functioning of basic physiological pathways. Such disease processes are fundamentally non-linear dynamic, being the results of an evolving interplay between homeostatic defense mechanisms and impaired physiological networks through space and time [88]. Since cell survival mechanisms under the control of stress response factors may also be those that trigger cell death depending on the pathophysiological context in which they operate [89] identifying the critical phases that, at the molecular, cellular, or system levels, are associated with the dynamics of ND processes and could modify the capacity of individuals to maintain function and resist ND is essential for clinical discovery and therapeutic developments, especially in the context of the growing needs for PM.

Recent years have witnessed significant advances in our understanding of how human diseases are routed in altered molecular and cellular networks. Several genetic alterations and pathophysiological mechanisms, mainly involving the amyloid-β protein precursor (AβPP) processing and tau related networks, are considered to be significant aspects in the pathogenesis of AD [90]. Such network derangements can cause either loss or gain of specific molecular functions and an increased formation of neurotoxic molecular species (e.g., toxic amyloid or protein aggregates) that can in turn adversely affect supra-cellular levels. Another important factor that should not be overlooked in the conceptualization of complex diseases is the crucial counteracting role of homeostatic networks. In this regard, the interest into the potential protective role of resilience factors against neurodegeneration (e.g., autophagy, proteostasis, endolysosomal networks, protein folding chaperone networks, disaggregates, and other stress-protective and clearance networks) is currently gaining momentum [90].

The causative pathways that lead to the onset of AD and its clinical phenotypes at the individual level are thought to consist of genetic/epigenetic susceptibility and/or protection coupled with a continuing dynamic interplay between altered brain networks and counteracting neural mechanisms of resilience. Integrative systems biology-based approaches are crucial to disentangling this intricate interplay. First, simple model organisms mimicking the main features of AD need to be developed in order to extensively apply different "omics" techniques. This approach may offer invaluable data to shed more light on the conserved pathways that modulate the onset and progression of AD, being ultimately useful for testing potential strategies that could delay and/or modify the natural course of disease [90]. However, the regulation of gene expression and pathway activity might differ between simple model organisms and humans, which calls for integrated use of simple model organisms and higher-order models such as mouse models and human cell models, e.g., induced-pluripotent-stem-cells coaxed into neurons or neurons obtained by direct conversion of fibroblasts [91].

New evidence from preclinical models needs to be duly replicated, with a special focus on subtle initial network alterations that can be visualized by neuroimaging, which could potentially become the targets of early therapeutic interventions [92–95]. Neuroimaging and biomarker data should be fully integrated and analyzed in a longitudinal manner through computational and integrative network biology tools within a systems biology-based framework. The increasing trend toward high-throughput techniques in AD research will generate multifactorial data that will require integration in a standardized, efficient, cost-effective, and secure manner. The vast amount of data generated will cause new challenges for data science, mainly in terms of data storage, processing, and mining. As we are entering into the "era of big and deep data" in AD, computational systems biology approaches are continuously being optimized in order to support the approximate modeling of biological systems [90].

A holistic systems biology-based research strategy in AD research will likely rely on generating large and rich data sets, applying multi-layer network approaches for integration and comparative assessments of different datasets, and reckoning on the information generated for discovery of novel disease markers and targets. A translational approach from preclinical studies to bedside (complemented by reverse translational approaches) will be required to integrate and implement fundamental aspects of the systems theory and the systems biology concept into clinical practice, i.e., translational systems medicine, in the upcoming future [96–99]. Key to the success of these approaches is the use of robust data integration methods. There is a large array of methods that enable complex data sets collected in experimental models of ND or human cohorts to be analyzed and integrated on a system level [100, 101]. Methods based on graph theory (that is net-

work approaches) such as spectral decomposition of the signal [102] weighted gene co-expression network analysis [103] and Bayesian causal inference [104] and those based on formal concept analysis [105] and tree induction [106, 107] likely hold strong promises for generating comprehensive *in silico* models that accurately select for biological rules, disease targets, and risk factors with potential for clinical exploitation.

APPLICATION OF SYSTEMS BIOLOGY IN AD COHORTS: THE EXAMPLE OF THE EUROPEAN PREVENTION OF ALZHEIMER'S DEMENTIA (EPAD) CONSORTIUM

Implementation of systems biology into clinical and research practice requires a number of steps. First, molecular tests and biomarkers for matching individuals/patients to clinical trials and/or targeted therapies will require continuous refinements and validation of high-throughput techniques, systems-level approaches, and computational tools. Second, all molecular tests to be used for AD, as well as all patient care-related molecular analyses, need to be performed using assays that are highly reproducible, accurate, and satisfy the FDA clinical trials guidelines, with adherence to principles of Good Clinical Practice (GCP) (available at http://www.fda.gov/regulatoryinformation/guidances/ucm122046.htm), the European Medicines Agency (EMA) (http://www.ema.europa.eu/ema/), and the European Clinical Trials Database (EudraCT) (https://eudract.ema.europa.eu/). In this scenario, the Alzheimer's disease neuroimaging initiative (ADNI) and the Dominantly Inherited Alzheimer Network (DIAN) will provide collaborative large-scale longitudinal data on AD associated autosomal dominant mutation carriers that will be invaluable to systematize and make explicit the translation of neuroimaging and biochemical markers into clinical guidelines. Third, the era of big and deep data generation and the availability of comprehensive repositories has brought the need for collaboration, sharing, integration, normalization, and analysis of both data and metadata, with the ultimate goal to make effective translational use of this new knowledge. In this scenario, several clinical trials may benefit from the holistic approach provided by systems biology. Among them, interest in the European Prevention of Alzheimer's Dementia (EPAD) program is gaining momentum.

The EPAD program [108] is a pan-European initiative that will establish a shared platform to design and conduct phase II Proof-of-Concept (PoC) clinical trials specifically aimed at developing new treatments for the secondary prevention of AD. To investigate different agents in the pre-AD population in the most efficient manner, a Bayesian adaptive design that learns from data accrued as the trial progresses will be used. Clearly disappointing results of recently completed phase III AD therapy trials may be explained by their exploratory (rather than confirmatory) nature, mostly caused by an incomplete exploration phase throughout phase II [109]. Hopefully, the EPAD program will be helpful to overcome previous pitfalls in the field by assuming that a correctly designed phase II trial can take several years to be completed. Other common issues that the EPAD Longitudinal Cohort Study (LCS) (available at https://clinicaltrials.gov/ct2/show/NCT02804789) will address include: 1) the high screen failure rates, 2) the unwillingness or inability to implement an adequate patient stratification, and 3) the lack of a pre-randomization run-in period. The EPAD LCS is expected to provide reliable disease models of the preclinical and prodromal periods of AD before the final implementation of a clinical trial. The EPAD LCS will be conducted in a large cohort of 5,000 subjects who had undergone a thorough assessment in terms of cognition [110, 111], neuroimaging, core CSF biomarkers ($A\beta_{42}$, total tau [t-tau], and hyperphosphorylated tau [p-tau]), clinical outcomes, and genotyping. Annual assessments will be performed with the goal of identifying different disease trajectories to provide an optimal stratification for trial inclusion. Risk stratification groups with similar biological underpinnings will be helpful to identify specific classes of subjects to be included (or excluded) from the clinical trial according to the PM paradigm.

The development of an EPAD site network across the European Trial Delivery Centers will be critical to the initiative success. Site certifications, continuing training, and commitment to the EPAD program is expected to reduce study site heterogeneity and will hopefully provide highly accurate estimates of treatment effects. Each TDC will assess approximately 200 research participants, of whom 100 will be included in the clinical trial. This effort is unprecedented, as previous clinical trials involved numerous centers (up to 200), each enrolling a handful of patients. Conversely, the traditional methodology will be overturned by EPAD, inasmuch as a few centers will enroll numerous patients.

In general, the correct implementation of phase III trials preliminary requires more robust phase II outcomes. The EPAD program will improve the study methodology, ultimately favoring an optimal disease modeling and a better patient stratification before embarking on phase III confirmatory trials. The EPAD LCS was started in May 2016 at six sites, with a total of 400 participants having already been recruited. Disease modeling work is expected to be introduced as soon as an enrolment goal of 500 subjects will be achieved. It is anticipated that the EPAD PoC Study Platform trial will begin in 2018.

SYSTEMS NEUROPHYSIOLOGY OF ALZHEIMER'S DISEASE: UNDERSTANDING NEUROPHYSIOLOGY AND NEURODYNAMICS BEHIND ETIOLOGY

During the last two decades, the neuroscience field has entered a rapid phase of expansion characterized by the development of a large proportion of methodologies allowing the recording of neural data obtained from a wide range of modalities, from metabolic pathways to optical imaging to functional magnetic resonance imaging (fMRI). These data are collected through different spatiotemporal domains (Fig. 5). Most of these techniques have been so far used one at a time [112, 113]. Recently, there is an attempt toward data integration in order to create comprehensive maps and record dynamic patterns across multiple levels of organization (neurons, circuits, systems, whole brain) and involving different domains of biology and data types (such as anatomical and functional connectivity, genetic/genomic patterns [112, 114]). This effort is in line with the new paradigm of systems neurophysiology aiming at integrating "big neuroscience data" recorded in a multimodal fashion to understand the role of the complex web of interconnections among several elements of large-scale neurobiological systems [115–118]. The ultimate goal of systems neurophysiology is to clarify how signals are represented within neocortical networks and the specific roles played by the multitude of the

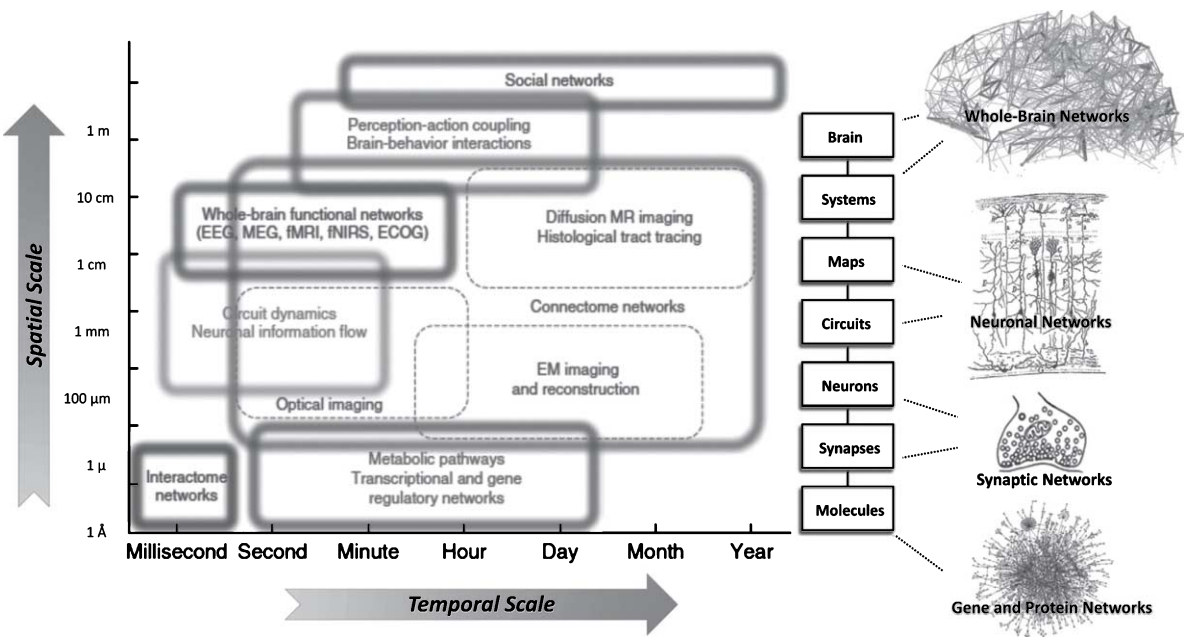


Fig. 5. Systems neurophysiology and network neuroscience: schematic representation of how structural levels within the nervous system integrate over multiple spatial and temporal scales. Network neuroscience encompasses the study of very different networks encountered across many spatial and temporal scales; however, the network ideas clearly extend down to the level of neuronal circuits and populations, individual neurons and synapses, as well as genetic regulatory and protein interaction networks. In network neuroscience and systems neurophysiology in general, the overall aim is to bridge information encoded in the relationships between genes and biomolecules to the information shared between neurons across to the brain level while integrating the additional information provided from the time dimension. This could eventually allow access to mechanistic understanding and models which faithfully reproduce and possibly predict both brain structure and function. Interestingly, above the single brain level, the social network level should still be considered a network neuroscience domain and, albeit with different measurement techniques, can be studied with the same paradigms with the aim to understand the larger "brain" that interacting brains give rise to (i.e., economies and cultures). Adapted from [112] and [609].

heterogeneous neuronal components. The new interdisciplinary field of network neuroscience proposes to overcome these enduring challenges by approaching brain structures and functions via an explicitly integrative perspective [112]. Here, we will present scientific advancements related to single methodologies utilized by system neurophysiology, within wider context of the PM paradigm in AD.

An increasingly important integrative component in this endeavor is connectomics the emerging science of brain networks, which comprises studies of both anatomical and functional brain connectivity, across modalities and methodologies. The rise of connectomics has triggered several national and international consortia devoted to mapping patterns of brain connectivity across large subject cohorts, including the Human Connectome Project funded by the U.S. National Institutes of Health [119]. These projects have pushed the boundaries of data sharing, neuroinformatics and computational analysis. Similar connectomics efforts are underway to track lifespan development [120] as well as address patient populations, including people with ND. To deal with the mounting volume of connectome data, the field is developing basic network science tools and methodology that can be applied to brain data [121]. So far, broad exploratory analysis has revealed a number of architectural principles that underpin macro- and meso-scale maps of brain connectivity, including modular organization and the existence of prominent hub regions. Much is still to be learned about the contributions of connectome architecture to human brain function and its role in pathophysiological processes. Systems neurophysiology in combination with connectomics and computational network models has great promise to illuminate the relation of structure to dynamics in brain networks as shown, for example, in recent findings on time-dependent functional connectivity as measured with non-invasive neuroimaging techniques.

CONTRIBUTION AND ROLE OF STRUCTURAL MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) is a widely, non-invasive, relatively non-expensive and versatile technology. Among MRI modalities, structural or anatomical MRI, using three-dimensional T1-weighted sequences, is the most widely used [122, 123] and validated [124, 125]. Structural MRI allows visualization and measurement of atrophy which is a macroscopic correlate of neurodegeneration, in particular of neuronal and dendritic loss. The progression of atrophy in AD approximately follows that of neurofibrillary tangles found in postmortem AD cases and described by Braak and colleagues [126] and Delacourte and colleagues [127]. Moreover, previous studies showed that structural MRI alterations correlate with tau deposition, as described by Braak stages, and CSF tau biomarkers [128]. On the contrary, not all structural MRI measures are well correlated to measures of Aβ deposition, and atrophy patterns do not follow those of amyloid deposition [129, 130]. Due to these reasons, it should be noted that brain atrophy in AD is descriptive of brain structural changes but not specific for underlying AD pathophysiology. Indeed, a given atrophy pattern can be associated with different pathophysiological processes. However, MRI atrophy measures are well correlated with cognitive and clinical functions [131, 132], and highly correlated with the concurrent rate of clinical decline [133–135]. Therefore, they constitute attractive tools to track disease progression and to monitor the effect of treatment.

Automated image analysis approaches allow measuring distributed patterns of atrophy across the whole brain, using either region-of-interest measurements, voxel-based maps of gray-matter or cortical thickness measurements [136, 137]. Machine learning algorithms applied to whole-brain atrophy maps can automatically identify patients with AD and thereby support diagnosis [138–141].

The most widely studied and accepted structural MRI marker of AD is atrophy of the medial temporal lobe [142, 143]. Assessment of medial temporal atrophy can be performed in clinical routine using visual scales [144]. However, such approach is observer-dependent and only semi-quantitative. On the other hand, fully-automated segmentation approaches provide objective, quantitative, volumetric measurement of hippocampal atrophy [145–149]. Hippocampal volumetry can discriminate AD patients from controls with high sensitivity and specificity [150]. Moreover, numerous studies have shown that patients with higher hippocampal atrophy are at higher risk of rapid cognitive decline [151–155]. However, atrophy of the hippocampus was found in other types of dementia, suggesting low specificity of this marker for the identification of AD [156, 157]. Recent developments of ultra-high field MRI (7 Tesla and higher) allow the study of anatomical alterations with an unprecedented level of detail. In particular, using

7T MRI, it is possible to distinguish between different cellular layers and anatomical subregions within the hippocampus. Its application in AD has demonstrated that hippocampal subregions and layers are differentially affected by atrophy [158, 159]. These advanced techniques have the potential to provide more sensitive measures than global hippocampal volumetry.

Another region of interest for AD is the basal forebrain cholinergic system (BFCS) since it represents the region with the majority of cholinergic nuclei efferent to the cerebral cortex [160, 161]. The measurement of BFCS nuclei has been developed and validated as a highly relevant and robust region of interest for automatic structural MRI assessment of atrophy rate of change from the preclinical to the clinical AD stages [160, 162–167]. Evidence indicates that the BFCS may even degenerate before medio-temporal lobe structures, as early as at the preclinical stage [163, 168]. In contrast to the hippocampal volume, the atrophy of BFCS was significantly correlated to *in vivo* brain amyloid load in AD and non-demented elderly individuals [169, 170]. Machine learning approaches based on whole brain atrophy patterns have been developed to predict the evolution of patients, in particular the progression to dementia of individuals with mild cognitive impairment (MCI) [171–173]. Nevertheless, most of these approaches have been validated on a single research dataset, most often provided by the ADNI. Therefore, their ability to generalize across datasets as well as their performance in a clinical routine context remain unclear and larger-scale validation studies are needed.

Its ability to track progression makes structural MRI also attractive to monitor the effect of treatment [29]. Of all outcome measures (including clinical, cognitive, and fluid biomarkers), structural MRI measures seem to have the highest measurement precision [135]. They are thus an attractive outcome measure for clinical trials, as well as to monitor the effect of treatment in a clinical context. It should be noted that different types of treatment seem to result in different effects on atrophy measures. In a randomized placebo-controlled trial, patients treated with donepezil, an acetylcholinesterase inhibitor, have a significantly lower rate of annual hippocampal atrophy and cortical thickness compared to those receiving placebo [174, 175]. Moreover, the treatment group demonstrated a significantly decreased annual rate of atrophy of the BFCS compared to MCI individuals that received placebo [176]. The BFCS complements hippocampal volumetry in assessing structural progression in AD and provides a promising outcome measure for clinical trials. Anti-amyloid therapies, however, seem to result in increased rate of atrophy [177]. Nevertheless, it may be hypothesized that such accelerated atrophy only occurs at the beginning of treatment, perhaps caused by a reduction in microglial activation associated with plaques, and that a reduction of atrophy may occur in the longer term. Overall, structural MRI remains an attractive tool to study the morphological effects of treatment, in particular if new molecules targeting other aspects of AD pathophysiology (e.g., anti-tau or neuroprotective treatments) become available. Furthermore, structural MRI plays an important role in monitoring safety of treatments. Indeed, microbleeds and transient cerebral edema (respectively called ARIAH and ARIAE) occur in some patients treated with active Aβ immunization [178].

In summary, structural MRI is an attractive marker for tailoring therapeutic interventions. Its most attractive features are its ability to precisely track cognitive decline, its potential for monitory the effect of treatment and to predict the evolution of patients. For prediction, the most promising avenue is that of machine learning approaches from whole-brain measurements. Such approaches require larger scale validation using multiple clinical routine cohorts. The integration of structural MRI analysis tools with other techniques such as those from functional MRI, electroencephalography (EEG), magnetoencephalography (MEG) or diffusion tensor imaging (DTI), in a multimodal fashion, will enable the investigation of temporal and topographical relationships between numerous pathological alterations and neurobiological systems related to AD. Such big data integration, will improve our understanding of the *in vivo* interacting pathophysiological mechanisms across brain related systems characterizing AD, as envisioned by the PM concept.

CONTRIBUTION AND ROLE OF DIFFUSION TENSOR IMAGING

Diffusion tensor imaging (DTI), which employs a Gaussian approximation to model the MR signal attenuation due to net water molecule displacements in a *de facto* restricted cellular environment. This technique has become the mainstream strategy for examining white matter (WM) microarchitecture, connectivity as well as integrity both in an investigative and in a clinical setting, and it has been

widely employed in studies focused on AD and MCI [179–181] as well as several other pathologies [182–185]. The apparent water diffusion tensor (which is termed *apparent* precisely because intracellular water diffusion is not truly free) can be estimated in brain parenchyma based on relatively fast echo planar imaging (EPI) techniques [186] which only pose moderate demand in terms of in-scanner subject time. From these tensor estimates, WM tract-specific orientation information can be obtained through deterministic (based on the orientation of the main DT eigenvector) or probabilistic approaches [187]. Also, model free tractography approaches exist, a promising development of which is constrained spherical deconvolution [188–191], which has lately been extended to incorporate multitissue models anatomically based filtering [188, 189] (Fig. 6). Further, scalar indices derived from the diffusion tensor are rotationally invariant and are well known to be sensitive, albeit not specific, indicators of microstructural alterations. The single tensor eigenvalues as well as mean diffusivity (MD – mean of eigenvalues) and fractional anisotropy (FA – normalized variance of eigenvalues [192]) can aid in quantifying fiber integrity through region of interest (ROI), voxel- or Tract-Based Spatial Statistics based approaches [180]. A decrease in FA (possibly accompanied by an increase of MD or other directional diffusivities) is typically the hallmark of unspecific bundle degeneration, as seen in AD and MCI [193, 194]. Importantly, correlations between DTI-derived indices in WM and AD disease severity have been reported [195, 196], suggesting that DTI measures may be used as indexes of disease progression. DTI may therefore provide unique information about WM integrity [66] in AD patients and MCI subjects. Indeed, several studies have demonstrated early WM changes within the parahippocampus, hippocampus, posterior cingulum, and splenium already at the MCI stage [197–200]. However, the majority of DTI studies indicate that the uncinate fasciculus, the entire corpus callosum and the cingulum tract are most involved in pathogenesis in both MCI and AD. In a recent study on AD and MCI subjects [201] the interpretation of a selective increase in FA in the MCI group was aided by the introduction tensor mode (MO) [202], a third invariant which distinguishes the type of anisotropy (planar, e.g., in regions of crossing or kissing fibers versus linear, in regions which exhibit one predominant orientation). This, in turn, led to the detection of a relative preservation of motor-related projection fibers crossing the association fibers of the superior longitudinal fasciculus in the early-stage MCI subjects before they degenerated to AD. Also, recent DTI data seems to point toward a reconstruction of the trajectory of progressive WM degeneration in AD as it spreads with aging. In agreement with this so called retrogenesis model (cortical regions that mature earliest in infancy tend to degenerate last in AD) it has been shown that WM abnormalities in specific brain regions such as prefrontal cortex WM, inferior longitudinal fasciculus, and temporo-parietal areas [180, 197, 203, 204] appear earlier. Also, DTI has been able to offer insight into asymptomatic "preclinical" at risk stages such as subjective cognitive decline, where DTI based scalar markers of diffusion properties were significantly associated with rates of cognitive decline and hippocampus atrophy at clinical follow up, with odds ratios up to 3 [205], and DTI indexes invariants were seen to be more sensitive than CSF biomarkers in predicting cognitive decline and medial temporal atrophy in subjective cognitive decline and MCI subjects [205].

Nevertheless, a recent meta-analysis indicates high variability in both the anatomy of regions studied and DTI-derived metrics [206], a partial contribution to which may be the intrinsic limits of the DTI techniques. Determining the most robust acquisition parameters and processing strategies for DTI for a multicenter setting is still an active area of research, and initial clinical and physical phantom data, i.e., scans obtained from a volunteer as well as a physical object with defined diffusion properties, suggest that the variability of DTI-based diffusion metrics across a range of MRI scanners is at least 50% higher than that of volumetric measures [207]. For prediction of conversion from MCI into AD dementia, DTI reached an accuracy of about 77%–95% at 2 to 3 years follow up [205, 208, 209] in monocenter studies, prediction accuracy for multicenter studies still needs to be studied. Also, all diffusion weighted imaging protocols suffer from the relatively low signal-to-noise ratio inherent in the necessarily fast EPI techniques. In this respect, the increase in signal-to-noise ratio afforded by moving to ultra-high field imaging (at, e.g., 7T) is somewhat counteracted by the rapid shortening of transverse (T2) relaxation times with increasing field strength and consequent signal loss. Nevertheless, while ultra-high field diffusion weighted imaging therefore poses significant challenges, improved distortion correction techniques [210] coupled with monopolar acquisition schemes which allow a significant (about 30%) shortening of echo times, and the

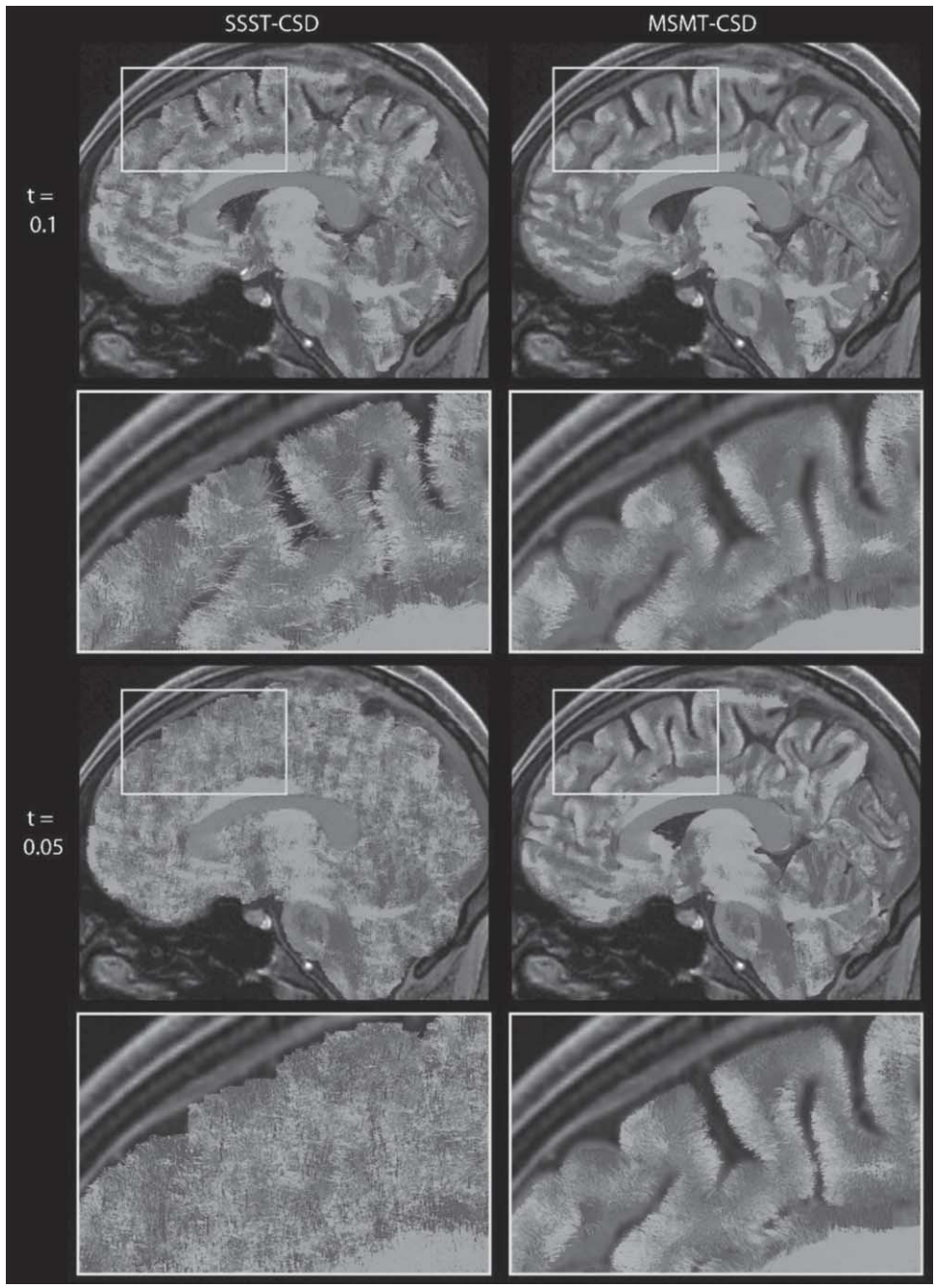


Fig. 6. Sagittal slab visualization of a fiber tractogram obtained from WM fODFs estimated with SSST-CSD (left) and MSMT-CSD (right) with different fODF amplitude thresholds (top, bottom). fODF, fiber orientation distribution function; MSMT-CSD, multi-shell, multi-tissue constrained spherical deconvolution; SSST-CSD, state-of-the-art single-shell, single-tissue constrained spherical deconvolution; WM, white matter. Reproduced with permission from [188].

additional use of simultaneous multislice excitation strategies [211] may allow *in vivo* diffusion-weighted imaging to finally advance toward sub-millimeter imaging at ultra-high field. Accordingly, *ex-vivo* studies have already defined WM lesions in aging and AD at 11.4T [212], and 7T imaging has been helpful in discriminating Parkinson's disease [213] and amyotrophic lateral sclerosis [214]. Finally, it is well known that the assumption of a Gaussian propagator (which is at the root of DTI) is insufficient in

regions with more intricate fiber architecture such as mixed tissue types and/or kissing or crossing fibers [215]. To this end, more advanced protocols such as Diffusion Spectrum Imaging [216], Diffusional Kurtosis Imaging [217–221], higher order tensor models [222], compartment models [223–225], and anomalous diffusion [226, 227], which can be optimized in order to enhance their suitability in a clinical setting [228], have been already been successfully employed in augmenting information about tissue degeneration in several ND, including AD [229–232].

Another avenue for DTI-based methodology is the construction and subsequent analysis of brain-wide maps of anatomical connections that can be summarized as structural networks or graphs [115]. Basically, these efforts proceed by first dividing the brain into a set of internally coherent gray matter parcels or regions (the nodes of the network) and then estimating the strengths of anatomical projections between these nodes (the edges of the network). While the reconstruction of such maps faces significant methodological issues, the resulting structural networks have been validated against classical histological techniques in non-human species. Human structural networks capture individual differences that relate to genetics [233] and various phenotypic variables, including indices of cognitive performance [234]. They also exhibit characteristic changes across the lifespan [120], during normal aging [235], and in the course of brain disorders [236]. For example, the loss of connectivity associated with the progression of AD results a loss of links between dense clusters of functionally-related regions and hence a decreased capacity for integration [237, 238].

CONTRIBUTION AND ROLE OF FUNCTIONAL MAGNETIC RESONANCE IMAGING

Using fMRI in a PM-based paradigm to tailoring therapeutics for patient treatment would be a very innovative approach from current methods to developing therapeutics for patients. The diagnosis and classification of patients would be based on clinical criteria, where a patient would be classified according to predetermined criteria. Implementation of a PM paradigm would use fMRI as a biomarker of functional brain changes that would be part of defining the patient's phenotype in combination with the other modalities. Thus, it would seek to integrate fMRI-based biomarkers within a systems neurophysiology context to provide an integrated picture of the patient's status [21]. The biomarkers within a systems neurophysiology approach would inform the treatment approach that a patient would receive. Given the complexity of AD and the other ND, the fMRI-based biomarkers would be integrated within a systems biology and neurophysiology approach with the other modalities (genetic, clinical, behavioral, cognitive, etc.) where the different biomarkers would reflect disease mechanisms, pathophysiology, clinical history and permit patient stratification for treatment [20, 21].

fMRI can be used to measure the vascular response to local neuronal activation due to stimuli or a cognitive task [239]. There are two broad approaches that may be utilized with fMRI data in defining PM-based biomarkers for AD detection and diagnosis—one would examine brain activation data in response to a stimulus or cognitive paradigm whereas another approach would examine the intrinsic connectivity networks measured using resting state fMRI. The first approach would lead to biomarkers that would be associated with the cognitive paradigm or stimulus class whereas examination of the intrinsic connectivity networks would provide a search for biomarkers over all brain networks.

In terms of a PM approach with tailoring therapeutics, the use of a cognitive task or stimulus would be a form of 'stress test' to a specific network, for example in asymptomatic at risk stages for preclinical and clinical AD, a memory task would typically activate the hippocampus, ventral- and dorsal-prefrontal regions, posterior cingulate regions [240–249], and a working memory task would primarily activate dorsal and ventral frontal regions and inferior and superior parietal regions [250–255]. A limitation of the cognitive paradigm approach is that the patient must be able to perform the task, and variability in task performance would alter the activation pattern [256–261]. An alternative approach in AD would be to implement cognitive paradigms outside of the memory domain that individuals may still be able to perform such as visual perception, attentional tasks, or passive stimuli [262–273]. The changes found using this approach would be applicable to patients that may be clinically more advanced, but also provides an approach to measure the 'downstream' effects of the pattern of disease-related neuropathology. Current studies examined the differences between patients and healthy controls or among different risk groups by quantifying the average difference between the groups, where the groups are defined by clinical-descriptive phenotypes or risk groups

based on genetics or family history. The proposed PM paradigm would instead examine the variability among the subjects to define phenotypes that are data-driven and may not necessarily reflect the underlying pathophysiology and clinical phenotypes. There is evidence of significant variability in brain activation from healthy status to MCI to mild AD stage, for example a using a face-name association paradigm, there was a nonlinear response in hippocampus, with higher activation in MCI subjects compared to healthy controls and AD dementia patients [242, 249, 274]. Similarly with the visual perception task the activation levels varied along the dorsal visual pathway as disease severity increased [262].

In addition to measuring brain function, one would need to integrate the above biomarkers with results from fMRI studies of the mechanisms of action of the potential therapeutics; most studies have examined cholinergic drugs over an extended treatment period in either MCI subjects or mild AD patients (see for example [273, 275–278]). Another potential approach to be used within a PM paradigm is to measure the effects of a single dose [279–282] and investigate the predictive power of the single dose over the effectiveness of the therapeutic strategy for the biomarkers-characterized patient. The single dose approach has the potential to inform the tailoring of the therapeutic intervention by providing information about potential medium to long term effects of any treatment.

The various fMRI-based paradigms described above would provide information about a specific brain network or set of brain regions and any data-driven approach would be limited to data from the brain network or regions activated during the task. An alternative approach utilizing fMRI would be to use whole-brain resting state fMRI to measure so-called resting-state networks or intrinsic connectivity networks (ICNs) [283–286]. These ICNs have been shown to be highly reproducible across individuals [287], exhibit characteristic dynamic fluctuations [288] as well as patterns of change across development, life span and in the course of brain disorders [236]. The topography of ICNs resembles other networks, such as those engaged during human behavior and cognition (for example, see [289–293]), derived from gene co-expression [294, 295], disease phenotypes and disease progression (for example [246, 296–303]), as well as brain activation level and cognitive performance (for example [293, 304–306]). The structure of ICN networks can be probed with a variety of network tools to reveal individual differences in their internal coherence and their mutual interactions. In combination with these advanced analytics, ICNs can potentially provide a rich set of biomarkers of brain function, including insights into which ICNs are specifically disturbed as a result of pathophysiology, and thus yield a more integrated perspective on system-wide changes within a patient. The tailoring of therapeutics could benefit from associations between biomarkers and the presence of the disease pathophysiology. Given the variability that is present in AD patients and MCI subjects, the ICN-based biomarkers and their relation to genetic profiles [68] may be able to provide an improved systems biology characterization of brain function. The use of ICNs for tailoring therapeutics still needs considerable development work, and there is currently only limited work on the effects of an AD-related drug on ICNs [307]. It should be noted that while the task-free design of resting fMRI lends itself to application in clinical cohorts, the sensitivity to motion artifacts and ongoing temporal fluctuations in the network structure of ICNs entail greater reproducibility as scan lengths are increased (for example, see [308]).

The potential of fMRI to assist in the PM-oriented targeting of therapeutics for AD patients is strong but also will require very significant development work. The integration of fMRI with the other domains such as genetics, cognition, clinical measures has so far mostly been attempted within a group analysis context, and a PM paradigm would need development of new statistical models to define potential therapeutic strategy on a single individuals basis [309].

CONTRIBUTION AND ROLE OF ELECTROENCEPHALOGRAPHY

Candidate topographic neurophysiological (neurodynamic) biomarkers of AD can be derived from resting state eyes-closed electroencephalographic (rsEEG) rhythms recorded in subjects relaxed in quiet wakefulness (eyes closed, no sleep) with their mind freely wandering [310]. These rsEEG markers are non-invasive, cost-effective, available worldwide, and repeatable even in severe dementia. They may probe the neurophysiological "reserve" in AD patients, as one of the dimensions of the brain reserve [311]. This neurophysiological "reserve" may reflect residual mechanisms for 1) "synchronization" of neural activity in a given cortical region and 2) the coupling of activity between nodes of a given brain

neural networks as a sign of functional cortical "connectivity" [310, 312].

RsEEG markers in AD at the group level reflect the neurophysiological reserve of the disease over time and after cholinergic therapy

Previous rsEEG studies using "synchronization" markers showed that compared with groups of normal elderly (Nold) subjects, AD groups with dementia (ADD) exhibited lower power density in posterior cortical alpha (8–12 Hz) and beta (13–30 Hz) rhythms [313–319]. There was also higher power density in widespread delta (<4 Hz) and theta (4–7 Hz) rhythms [320–325]. Finally, ADD, dementia due to Parkinson's (PDD), and dementia with Lewy bodies (DLB) groups were characterized by abnormally lower posterior alpha source activities [326]. The effect was dramatic in the ADD, marked in the DLB, and moderate in the PDD [326]. There were also abnormally higher occipital delta source activities with dramatic effects in the PDD group, marked in the DLB group, and moderate in the ADD group [326].

Concerning "connectivity" markers, ADD groups were characterized by abnormally lower spectral coherence in alpha and beta (13–20 Hz) rhythms between posterior electrode pairs [316, 327–339]. These effects were observed in temporo-parieto-occipital electrode pairs in some studies [316, 327, 333, 337] and in frontocentral electrode pairs in others [329, 332, 340]. Other studies reported either a global decrease [327, 334] or increase [337, 341] of delta and theta coherences between electrode pairs in ADD groups. Another investigation pointed to a complex topographical pattern of coherence increase and a decrease in those groups [342]. Alternative techniques of "connectivity" unveiled a decrement of synchronization likelihood between electrode pairs in frontoparietal alpha rhythms in ADD and its prodromal stage of amnesic MCI [319, 343]. Finally, there were reduced cortical connectivity and "small-worldness" in ADD groups as revealed by graph theory indexes [344–347].

RsEEG rhythms deteriorate across time (e.g., about 12–24 months) in groups of aMCI subjects and ADD patients (see for a review [348]): 1) increased delta-theta and increased alpha-beta power density at parieto-occipital electrodes [349]; 2) increased theta power density, decreased beta power density, and decreased mean frequency at the temporal and temporo-occipital electrodes [316, 350, 351]; 3) increased delta and increased alpha 1 in parieto-occipital sources [352, 353]; and 4) reduced cortical connectivity as revealed by graph theory indexes [347].

In groups of ADD patients, acetylcholinesterase inhibitor drugs (i.e., enhancing the cholinergic tone) showed beneficial or protective effects in delta [320, 354–356], theta [321, 356, 357], and alpha rhythms [355, 358]. When observed at short-term, these effects predicted longer-term therapy efficacy [357, 359, 360] (for a review, see [352]). However, some contradictory findings suggest future more controlled cross-validation studies [361, 362].

Abnormal posterior cortical delta rhythms in ADD patients might reflect an upregulation of their generation mechanisms in quiet wakefulness, possibly due to cortical blood hypoperfusion and synaptic dysfunction in the same regions [363–366] and atrophy in the posterior cortex [312, 352, 367–369]. Furthermore, reduced posterior cortical alpha rhythms in ADD subjects might be due to an unselective tonic cortical excitation in populations of cortical pyramidal, thalamo-cortical, and reticular thalamic neurons generating those rhythms [370–372]. Such cortical over excitation might induce a background noise in the neural information processing interfering with vigilance and cognition [310].

RsEEG markers in AD at the individual level: Classification accuracy and predictions

RsEEG markers allowed the discrimination of ADD patients from Nold individuals and others with neurodegenerative dementing disorders such as PDD and DLB persons. Global delta and alpha coherences between electrode pairs successfully classified ADD compared with DLB people with 0.75–0.80 (e.g., 1 = 100%; [373]). Furthermore, twenty discriminant scalp rsEEG power density and coherence variables showed a classification accuracy of 0.90 in the discrimination of ADD versus Nold and ADD versus PDD subjects [374]. Another study in small populations of ADD, PDD/DLB, and frontotemporal dementia (FTD) patients reached a classification accuracy of 1.0 using 25 discriminant scalp rsEEG power density and functional cortical connectivity (i.e., Granger causality) variables [375]. In another study, combining quantitative rsEEG variables (including those of functional cortical connectivity) with neuropsychological, clinical, neuroimaging, cerebrospinal fluid, and visual EEG data reached "only" a classification accuracy of 0.87 in the discrimination between ADD, PDD, and DLB

persons [376]. Concerning cortical source space, resting state delta and alpha sources classified Nold subjects versus ADD/DLB/PDD patients and ADD versus PDD patients with 0.85–0.90 [326]. Milder classification effects were observed in PDD and ADD individuals with MCI [377]. RsEEG markers predicted cognitive decline in aMCI individuals at about 6–24 months (see [348] for a review). The main effects are summarized as follows: 1) combined alpha-theta power density and mean frequency from left temporal-occipital regions [316]; 2) anterior localization of alpha sources [315]; 3) high temporal delta sources [378]; 4) high theta power density [379]; and 5) low posterior alpha power density [380].

Concluding remarks on EEG implementation

Overall, it is suggested that resting state cortical delta and alpha rhythms might unveil more compromised neurophysiological reserve in AD, at the group and the individual level. These rsEEG markers predicted and tracked the AD progression as neurophysiological endpoints for therapeutic interventions. Future multi-centric longitudinal studies should provide a large open access database for a systematic comparison of rsEEG markers of "synchronization" and "connectivity" markers for a better definition of "neurophysiological reserve" for clinical applications and research.

CONTRIBUTION AND ROLE OF MAGNETOENCEPHALOGRAPHY

Magnetoencephalography (MEG) allows recording the magnetic signals of the order of 10^{-12} Teslas, which are produced at the scalp surface by the activity of neuronal assemblies. It may provide information complementary to EEG for uncovering new neurodynamic biomarkers of AD, particularly in its very early asymptomatic at risk and preclinical stages, therefore before the prodromal and clinical stages.

MEG can be used to investigate cognitive functions in a way very similar to EEG. With this approach, impaired brain functional activities were characterized in AD and MCI stages during memory tasks for instance. Walla and colleagues [381] used a recognition memory task in which they manipulated the depth of encoding of verbal information. They showed alteration of temporo-parietal event-related responses to old—previously encoded—versus new items in AD patients relative to controls, after deep encoding. The mismatch negativity (MMN) was also shown to be a potential AD marker. The mismatch negativity is a well-known component of the event-related potential response, which is associated with the detection of deviant stimuli in a stream of standard, repeated stimuli—classically in the auditory modality, hence allowing the assessment of the quality of sensory processing, memory, and predictive coding [382, 383]. Its magnetic counterpart, the MMNm, was shown to be delayed in latency in AD compared to healthy elderly controls [384] (see also [385]). Most interestingly, using memory tasks in preclinical stages of AD, e.g., in *APOE ε4* carriers, some studies pointed to the capacity of MEG for revealing neurophysiological markers of subjects' decline, potentially predictive of pathology emergence [386, 387]. In sum, MEG can be used in the same way as EEG to investigate cognitive functions during various task performance; both these methods provide highly convergent and temporally detailed data on information processing and cognitive functions in normal and pathological aging.

However, the most unique potential of MEG for uncovering pathophysiological mechanisms and providing new neurodynamic biomarkers in the field of AD may lie in the study of functional brain networks, particularly of resting state networks (for review, [388]). As mentioned above, fMRI studies have shown that, in the absence of task demand, the resting brain exhibits spontaneous and highly structured, often oscillatory, fluctuations in activity [389]. MEG and EEG provide a richer view of these networks in the time and frequency domains [390–395]. Resting state networks are usually studied using time-frequency decomposition of MEG (or EEG) signals. This allows identifying a rich set of resting state networks in distinct frequency bands (e.g., [390, 392, 393, 396]). It was shown that AD patients show altered resting state network activity. This was revealed at the level of oscillatory activity characteristics, pointing to an overall slowing of brain rhythms with particular abnormalities in the delta (<4 Hz) and beta (∼20 Hz) frequency ranges [397–402]. Moreover, alteration of resting state networks, correlated with memory impairment, was recently shown using a graph-theoretical approach applied to neuromagnetic data [403]. Important questions are: When do these changes emerge in the course of the disease and which changes are predictive of or specific for the development of molecular and clinical AD? There is particular potential in EEG and MEG methods to provide such a surrogate biomarker for clinical outcome. Moreover, there is evidence that some MEG mark-

ers of functional brain networks may be predictive of the conversion from MCI to AD dementia [397, 400, 404].

On a practical note, it is important to underline that resting state studies have the advantage to be particularly adapted for elderly patients, because they require no cognitive effort and require relatively modest data acquisition time. It is worth mentioning that MEG, in comparison to most EEG systems, requires only a short time of subject's preparation for recording. The whole-head MEG systems that are available at present comprise about 300 sensors that are fixed in a rigid helmet. After head shape numeration and the installation of a few reference sensors, individuals are comfortably seated with their head placed in the helmet. The installation time takes as little as 20 minutes. Moreover, the total "innocuity" of MEG allows close follow-up and detailed longitudinal assessment of disease progression.

The recent development and promising results of neuromagnetic imaging methods has led to the Magnetoencephalography International Consortium of Alzheimer's Disease (MAGIC-AD) initiative. This initiative aims at advancing the use of MEG for AD and pre-AD research, combining data from resting state and simple memory and MMN tasks, in a multicentric study [405]. While still in its burgeoning with regard to clinical applications, MEG has the potential to provide new tools for patient stratification, in order to better target patient population for clinical trials, and for treatment evaluation [406, 407], and to shed new light on the neurodynamic pathophysiological mechanisms of AD. It allows to foresee the identification of individualized signatures of disease progression in the form of temporal profiles of early adaptive, compensatory, and decompensatory brain network changes. Moreover, it is clear that the full power of MEG will come from its combination with other methods to allow multimodal assessment of individuals and IDM of multi-modal big data. For example, the combination of genetic data, such as the APOE polymorphism characterization with MEG resting state analysis has revealed promising in identifying MCI subjects at high risk of conversion to AD dementia as well as asymptomatic subjects at high risk of developing significant cognitive deterioration [408]. Multifactorial characterization of MCI subjects, including neuropsychological assessment, structural and functional brain measures, APOE genotyping, demonstrated very high sensitivity and specificity for predicting conversion to AD [409].

In conclusion, the advances in the characterization of the dynamics of functional brain networks based on MEG stands the chance to provide new insights into the pathophysiological mechanisms of AD. In doing so, it shall constitute a powerful tool to bridge the gap between what is known from the cellular and molecular pathways of the disease—its start and its progression—and the cognitive dysfunctions constituting its clinical and behavioral hallmark. This is likely to be key for developing new biomarker-guided targeted treatments and PM, based on the characterization of the individual genetic patterns and pathophysiological pathways towards neurodegeneration and dementia.

CONTRIBUTION AND ROLE OF NEUROMODULATION

Neuromodulation refers to forms of more or less invasive targeted and reversible electrical stimulation of discrete brain regions; it usually assists, but not replaces, traditional pharmacological treatments, with the aim to induce long-lasting changes of firing neural properties, both in the target region and connected networks, thereby modifying behavior or diseases' symptoms. Therefore, neuromodulation fits well with the broad paradigm of PM that is the customization of healthcare tailored on the individual patients' demands and disease's pathophysiology.

Invasive neuromodulation in AD

Neuromodulation through deep brain stimulation (DBS) is an emerging opportunity in AD, being already an established therapy for advanced neurological and psychiatric diseases [410]. Several subcortical and cortical targets of stimulation have experimentally shown improvements in learning and memory, reinforcement of synaptic strength and restoring of physiological patterns of oscillatory brain activity, especially in the theta band, a rhythm that is functional to memorization [411]. DBS of the entorhinal cortex [412] enhanced memory of spatial information when applied during learning. DBS of the nucleus basalis of Meynert was studied in six patients with mild to moderate AD in a 12-month pilot study [413]. DBS was well tolerated and 4 of 6 patients were considered stable or improved at 12 months based on cognitive scores. The fornix, a deep WM tract interconnecting hippocampus with mammillary bodies, and a central node of the Papez circuitry which is integral to memory function [411],

has been the most investigated, human DBS target for AD [414–417].

A 12-month follow-up of the first implanted 6 patients in the bilateral fornix showed a possible slowing of cognitive decline in some of them, accompanied by increase of metabolism in memory-related neural network structures [418], and by a reversal of the usual hippocampal atrophy found in AD [416]. These promising results prompted the first multicenter, 12-month, double-blind, randomized, controlled study of bilateral DBS of bilateral fornix in 42 patients with mild probable AD [419, 420]. The study showed no differences between those patients who received stimulation compared to controls who were not stimulated in cognitive measures. However, patients who received stimulation showed an increase in glucose metabolism in pre-selected brain regions at 6 and 12 months whereas those who were not stimulated showed decreased metabolism as expected. In a *post-hoc* regression analysis age was associated with outcome. Patients with late onset disease ($\geq$65 years old) receiving stimulation showed a slowing of decline in cognitive measures when compared to those not stimulated. Improvement in glucose metabolism in this subgroup was greater in magnitude compared to the group as a whole. Stimulation of the fornix appeared to be safe. The overall perioperative adverse effects of the procedure, despite the cortical atrophy and the trans-ventricular trajectories of the electrodes towards the deep target, were comparable in DBS in other ND and there was no evidence of mortality or neurological morbidity at three months from the implant [419].

Non-invasive neuromodulation in AD

A different, non-invasive yet still experimental in AD, research approach for neuromodulation is the targeting of neocortical regions relevant to AD pathophysiology, through the scalp by applying repetitive transcranial magnetic stimulation (rTMS) or weak currents via transcranial direct current stimulation (tDCS), in repeated daily sessions of stimulation [421]. Mechanisms of action are different, as rTMS makes cortical neurons to fire trans-synaptically [422], while tDCS shifts the level of their firing probability in a polarity-dependent manner [423]. Both stimulation techniques induce controllable excitatory or inhibitory after effects: high-frequency rTMS and anodal tDCS generally increase cortical excitability, while low-frequency rTMS and cathodal tDCS do the opposite [424, 425]; these effects are either local or involve the cortico-subcortical network to which the targeted region belongs [426]. In case of AD, the mere "stimulation" of a cortical target, even if prolonged for several daily sessions, does not help so much in preventing the decline of memory and other cognitive functions [421]. However, there are few controlled studies for rTMS in AD and even less for tDCS, for a total of a few dozens of patients treated so far [421]. What is emerging as a possible role for non-invasive neuromodulation is the coupling of stimulation with cognitive therapy, with the aim to promote plastic associative learning mechanisms to synergically improve the effects of cognitive rehabilitation only [427–429]. This approach, while still in need of quantitative characterization [430–432] seems promising only in mild AD, when the severity of neurodegeneration makes still available a residual neural substrate to possibly intervene on [433].

From the bench to the patient: A future way of non-invasive neuromodulation?

Physiological cerebral activity is composed of oscillatory activity across a wide range of frequencies, ranging from 0.05 up to 500–600 Hz: oscillations in the 30–80 Hz range are known as "gamma" activity. A relative attenuation of gamma activity is a consistent finding in patients with AD [315]. Moreover, dysregulation of hippocampal theta/gamma coupling may precede amyloid deposit activity in animal models of AD [434]. A seminal recent study in pre-symptomatic and amyloid pre-depositing AD mice, showed that exogenously-induced flickering lights oscillating at 40 Hz reduce Aβ concentrations and amyloid plaques, as well as tau concentrations, in a mouse model of AD [435], preventing subsequent neurodegeneration and behavioral deficits, thus suggesting that gamma induction may represent a novel therapeutic approach for AD. This opens translational perspectives, as the possibility of modulating gamma activity in humans, potentially leading to the same beneficial effects observed in mouse models. The possibility of modulating brain oscillatory patterns in AD patients has been recently shown, with EEG changes in brain connectivity in the gamma band following the administration of antiepileptic drugs [436].

A viable way to interact with brain oscillations is transcranial alternating current stimulation (tACS), where low intensity (max 2 mA) alternating sinusoidal currents are applied via scalp electrodes. Due to the safety [437] and controllability (in terms of

stimulation frequency and the possibility to target almost any cortical region) of the procedure, tACS has gained consensus as one of the most promising techniques to modulate brain oscillations in the healthy and pathological brains. Empirical evidence using neurophysiological markers, demonstrate that tACS modulates brain oscillatory activity via network resonance, suggesting that a weak stimulation at a resonant frequency could cause large-scale modulation of network activity and amplify endogenous network oscillations in a frequency-specific manner [438–441]. The application of tACS in the gamma band (specifically 40 Hz) has been shown effective in transiently modulating various abilities in humans, including those related to higher-order cognition [442, 443] and sensorimotor performance [444]. The repeated administration of tACS in AD patients, if individually tailored on cortical regions with higher concentration of Aβ, might constitute a timely, disease-transforming, personalized therapeutic application worth to be tested in patient populations.

CONTRIBUTION AND ROLE OF POSITRON EMISSION TOMOGRAPHY

Positron emission tomography (PET) has the potential to make a major contribution to selection for treatment in AD. This is of particular interest at very early asymptomatic stages of the disease, when clinical symptoms are still absent. In addition, it may also turn out as important at later stages as it is increasingly being recognized that several distinct pathophysiological processes can contribute to the development and manifestation of first symptoms and dementia. They vary considerably among patients, and one would therefore want to target the leading cause in individual patients.

At preclinical or prodromal disease stages identification of fibrillary amyloid deposits by PET currently is of obvious importance as an approved imaging biomarker for clinical trials. Use of a conservative cut-point has been suggested to minimize inclusion of elderly subjects with beginning amyloid deposition but without subsequent worsening [445]. Depending on a positive outcome of trials, amyloid PET might become a theragnostic procedure to select patients for anti-amyloid treatment.

In individuals with manifest dementia, differential diagnosis between AD and other diseases, such as FTD and vascular dementia, is important for selecting symptomatic treatment. ^{18}F-2-fluoro-2-deoxy-D-glucose PET (^{18}F-FDG-PET) has repeatedly been demonstrated to provide reliable differentiation between AD and FTD [446]. Beyond its relevance in the differential diagnosis, ^{18}F-FDG-PET is a topographic marker of AD that can be used to measure disease progression and help identifying clinical subtypes [447]. Thus, it has a mediational effect between the neuropathological hallmarks of the disease (neurofibrillary tangles and Aβ) and the cognitive symptoms [448]. It has also been used successfully to study mechanisms underlying cognitive reserve, which delays the onset of dementia [449]. Identification of *in vivo* AD pathology has also proven to be relevant in disease identification. Indeed, some AD clinical phenotypes can be underlain by several neurodegenerative disorders (e.g., primary progressive aphasia, corticobasal syndrome), including the classical amnestic AD [450]. In such cases amyloid PET can identify fibrillary amyloid as an indicator of AD. Fibrillary amyloid can also coexist with other pathologies, which is frequently the case in patients with DLB and vascular dementia (which might be termed mixed dementia), but is also possible with FTD and may possibly contribute to more rapid progression [451, 452]. Thus, if anti-amyloid therapy did eventually show clinical benefit in AD patients, patients with non-AD dementia and positive amyloid PET might also benefit.

Among the large variety of possible pathophysiological contributors to AD, many are accessible by specific PET tracers. The most prominent are fibrillary tau deposits. The current generation of PET tau tracers has been demonstrated to reflect the pathological staging of tau deposits in AD, but there is also evidence of some off-target binding that complicates the interpretation of scans. Next generation tracers are being developed to overcome these limitations [453].

Neuroinflammation is another major factor which has been shown to accelerate disease progression. It is associated with activation of microglia, which can be imaged by PET using the translocator protein (TSPO) tracers. ^{11}C-(R)-PK11195 has been the first of those, and in spite of some limitations due to a relatively high level of non-specific binding is still widely used. A large number of second generation tracers with higher specificity has been developed but their binding is subject to a genetic polymorphisms that blurs the advantage of these tracers [454]. Nonetheless, beyond these limitations, the development of these tracers could provide relevant biomarkers and offer new insights in the variability of evolution of AD [455]. There are also tracers for imaging of

astrogliosis, and markers for cytokines and inflammatory endothelial changes are being developed. Further translational research will investigate the molecular characteristics and the effects of targeted interventions on microglial and astrocytic activation. Deficits in cholinergic transmission play a major role for deficits in memory and attention in patients with dementia. Tracers have been developed for nicotinic and muscarinic receptors, for vesicular transporters and acetylcholinesterase. Clinical studies have provided preliminary evidence that such tracers could be used to identify responders to acetylcholinesterase inhibitor therapy, and further research into this issue is required [456].

There are well established single photon emission computed tomography (SPECT) and PET tracers for identification in dopaminergic transmission, which is most severely affected in DLB. This is providing a useful diagnostic tool for differentiation between AD and DLB, while research is ongoing to identify the cognitive deficits associated with that deficit and potential targeted therapeutic interventions [457].

There is also current research into PET imaging of glucose energy metabolism, mitochondrial damage, glutamatergic and GABAergic dysfunction, blood-brain barrier damage and defects in transcriptional regulation and protein synthesis. They may play an important role in AD pathophysiology and offer windows for targeted intervention.

In conclusion, there is a huge potential of PET to contribute development of the PM paradigm in AD. Currently, amyloid imaging has been progressed most as a biomarker in clinical trials towards that goal. ^{18}F-FDG-PET and tau-PET imaging are also involved in multiple trials, while a large variety of other tracer for specific targets in AD pathophysiology are still at earlier stages of translational research.

CONTRIBUTION AND ROLE OF RETINAL IMAGING

Over the past three decades, growing evidence indicates that AD is not confined to the brain but also affects the eye. Patients with AD and subjects with MCI experience a wide spectrum of visual deficits [458–464], sleep disturbances [465–471], and ocular abnormalities [466, 472–489]. Historically, these visual and circadian rhythm disturbances were attributed to pathology in the brain yet are now being revisited and explored as a potential direct outcome of ocular pathologies. Among ocular tissues, studies have shown that the retina is massively impacted by AD [466, 472, 474–479, 482, 484, 486, 487, 490–507]. The retina of MCI subjects and AD patients displays a host of abnormalities including nerve fiber layer (NFL) thinning, optic nerve and retinal ganglion cell (RGC) degeneration, macular volume changes, retinal angiopathy involving reduced blood flow and vascular structural alterations, astrogliosis, and abnormal electroretinogram patterns [472]. Given these findings, it is no surprise that attention has begun shifting towards the neuroretina as a site of AD manifestation.

As a CNS tissue derived from the embryonic diencephalon, the retina shares many structural and functional features with the brain [508], including the presence of neurons, astroglia, microglia, pericytes, microvasculature with similar morphological and physiological properties, and a blood barrier [509–511]. Axons of the optic nerve directly connect the retina and brain, facilitating vesicular transportation of AβPP synthesized in RGCs [512]. Further, retinal neurons and glia secrete proteins associated with the amyloid cascade including γ-secretase, BACE1, Apolipoprotein E, and clusterin [511, 513, 514]. However, the skull-encased brain is shielded by bone, whereas the retina is accessible for direct, non-invasive high-resolution imaging.

The converging evidence denoting retinal abnormalities related to nerve degeneration and vascular changes, common to various neurological and ocular diseases, have long been described in MCI subjects and AD patients. Yet, the AD-specific pathophysiological hallmark, Aβ plaques, was only recently identified in postmortem retinas of AD patients and early-stage cases [490]. Subsequent studies corroborated these findings of retinal Aβ deposits and further indicated the presence of p-tau in retinas of AD patients [466, 485, 489, 515, 516]. These studies provided evidence for elevated retinal $A\beta_{40}$ and $A\beta_{42}$ peptides using biochemical assays on whole retinal extracts and revealed diverse retinal Aβ plaque morphology in flatmounts, often associated with blood vessels or co-localized with sites of cell degeneration (Fig. 7A-H) [466, 485, 489, 490, 515, 516]. Recent data showed that retinal Aβ deposits were found in clusters and frequently mapped to peripheral regions in the superior quadrant in AD patients (Fig. 7C, F). The load of $A\beta_{42}$-containg retinal plaques in the superior quadrant was substantially elevated by 4.7-fold in patients compared to age- and gender-matched controls (Fig. 7C, D) [485]. While two groups were unable to detect Aβ or p-tau in the

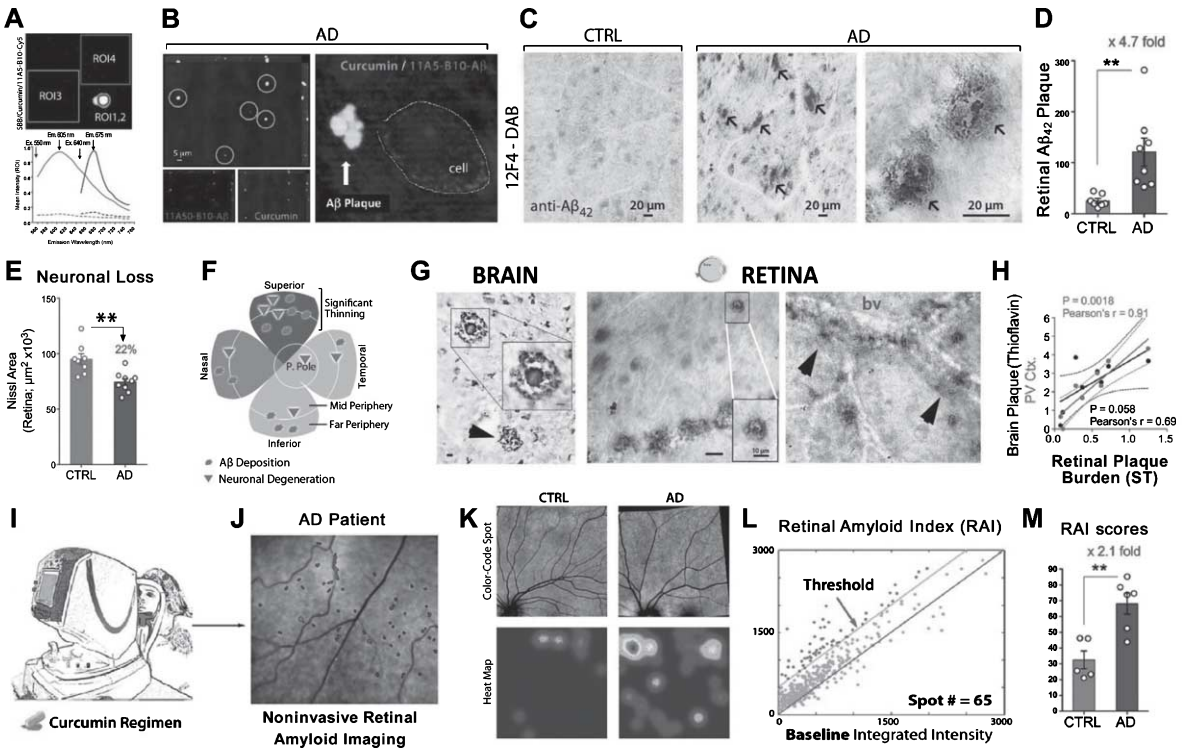


Fig. 7. Retinal amyloid imaging: from histological examination to clinical trials. A) Spectral analysis of Aβ plaque in AD human flatmount retina via specific curcumin labeling. Representative image and spectra curves of retinal Aβ plaque double-labeled with curcumin [region of interest (ROI) 1; orange line] and anti-Aβ_{40} antibody-Cy5 conjugate (ROI2; purple line) and corresponding background areas (ROI3 and ROI4; dashed lines) at excitation wavelengths of 550 nm (for curcumin spectra) and 640 nm (for Ab-Cy5 conjugate). Sudan black B (SBB) was applied to quench autofluorescence. Peak emission wavelengths captured for the same individual Aβ plaque (605 nm for curcumin when bound to Aβ plaque and 675 nm for anti-Aβ Ab conjugated Cy5) are distinct, indicating specific fluorescent signals for each fluorochrome and signifying the detection of Aβ plaque by curcumin. B) Representative z-axis projection images of flatmount retinas from AD patients. Retinal Aβ plaques (yellow spots) co-labeled with curcumin (green) and anti-Aβ_{40} monoclonal antibody (11A50-B10; red) are detected. Analysis included definite AD ($n = 8$), probable/possible AD ($n = 5$), and age-matched controls ($n = 5$). High-magnification image (right) showing an extracellular Aβ plaque. Images A-B are adopted from [490]. C) Representative microscopic images from flatmount retinas of a healthy control individual (CTRL; 71 years) and a definite AD patient (74 years) stained with anti-Aβ_{42} C-terminal-specific antibody (12F4) and visualized with peroxidase-based labeling. High-magnification image showing different Aβ_{42} plaques including classical morphology. Analysis included definite AD patients ($n = 5$) and matched controls ($n = 5$). Images reproduced from [466, 472]. D) Quantitative analysis of retinal Aβ_{42}-containing plaques (12F4-immunoreactive area) in the superior quadrant shows a significant increase in AD patients versus matched controls. E) Quantitative Nissl$^+$ neuronal area in retinal cross sections indicated a significant reduction in AD patients compared to CTRLs, which is associated with retinal neuronal loss. D, E) Data reprinted from [485] ($n = 23$ AD patients and $n = 14$ controls). F) Retinal flatmount illustration demonstrating the geometric distribution of pathology in AD retina by quadrant, with more consistent findings of nerve fiber layer thinning, neuronal degeneration and retinal Aβ deposits mapped to peripheral regions of the superior quadrant. Adopted from [472]. G) Representative images of a frontal cortex section and a flatmount retina from AD patients stained with 12F4 monoclonal antibody (brown) showing different Aβ_{42} plaque morphology including classical plaques (inserts). Clusters of Aβ_{42}-containing plaques are often associated with blood vessels (bv; right image). H) Correlation analyses using Pearson's coefficient (r) test between retinal 12F4$^+$-plaque burden in the superior-temporal (ST) quadrant and cerebral plaque burden (Thioflavin-S staining) in a total of seven brain regions (Brain; black) and in the primary visual cortex alone (PV Ctx.; green) in a subset of AD patients and matched CTRLs. I, J) Illustration displaying non-invasive retinal amyloid imaging using Longvida® curcumin and a modified scanning laser ophthalmoscope in human trials. K-M) *In vivo* retinal imaging in AD patients and age-matched controls. K, L) Increased curcumin fluorescent signal (red dots) in superior hemisphere in AD patient versus CTRL. Color-coded spot overlay images: red spots are above threshold and considered curcumin-positive amyloid deposits; green spots exceed 1:1 reference but not threshold; blue spots fall below reference. Heat map images with red spot centroids (lower panel) showing regions of interest with more amyloid plaques in the retina. L) Automated calculation of retinal amyloid index (RAI). Blue line is 1:1 reference; green line represents the threshold level, determined at 500 counts and above; red spots are above the threshold. The same automated image processing and analysis was applied on all human subjects ($n = 16$). M) RAI scores showing significant increase in AD patients compared to age-matched CTRLs. G-M) Republished with permission of American Society for Clinical Investigation from [485]; permission conveyed through Copyright Clearance Center, Inc. Group means and SEMs are shown. $^{**}p < 0.01$, unpaired two-tailed Student's *t*-test.

human AD retina [489, 517], they relied on analysis of cross sections prepared from narrow strips spanning horizontally from nasal to temporal quadrants, regions scarce in Aβ pathology. In contrast, a recent study provided in-depth characterization of retinal Aβ deposits in larger cohorts of definite AD patients via scans of large retinal areas in flatmounts and in cross sections derived from geometrical regions abundant with Aβ pathology [485]. The discovery of classical, dense-core (compact), and neuritic-like plaques in these patients, albeit smaller in average size compared to plaques in the brain, along with neurofibrillary tangles, $A\beta_{42}$ fibrils, protofibrils, and structures resembling oligomers, suggests that the specific signs of AD are shared between the retina and the brain (Fig. 7G). A correlation analysis in a subset of patients has validated positive relationships between retinal and respective cerebral Aβ plaque burden, with a tighter association to plaques in the primary visual cortex (Fig. 7H) [485]. Notably, retinal regions in AD patients where abundant Aβ pathology was detected— the periphery of the superior quadrant and the innermost retinal layers—also showed a significant decrease in retinal neuronal cells (Fig. 7E, F), in agreement with previous studies showing a marked RGC loss and NFL thinning in the superior quadrants [466, 476, 484, 491, 498, 502, 518, 519]. A recent clinical study identified circadian abnormalities in AD patients along with a significant loss of melanopsin RGCs (mRGCs), photoreceptors known to drive circadian photoentrainment [520], and discovered Aβ accumulation within and around these degenerating cells. The loss of mRGCs may therefore result from their increased susceptibility to toxic Aβ forms and offers a plausible retina-based explanation for sleep disturbances in AD [466].

In line with the above findings, numerous studies examining the retina of transgenic and sporadic animal models of AD have reported Aβ deposits, vascular Aβ, p-tau, and paired helical filament-tau (PHF-tau), often in association with RGC degeneration, local inflammation (i.e., microglial activation), and impairments in retinal structure and function [472, 485, 490, 515, 516, 520–537]. These investigations, which included a variety of transgenic rat and mouse models (ADtg) as well as the sporadic rodent model of AD, O. degus, demonstrated abundant Aβ deposits, mainly in the GCL and NFL [490, 516, 521, 525, 528, 530, 533]. Furthermore, several publications have described positive responses to therapies in reducing retinal Aβ plaque burden in ADtg mice, often reflecting the reactions observed in the respective brains [490, 524, 527, 528, 532, 536].

To visualize retinal Aβ pathology in live subjects, a non-invasive retinal amyloid imaging approach was initially developed in ADtg mice, utilizing curcumin as a fluorescent probe [490, 527]. Curcumin is a natural and safe fluorochrome that crosses the blood-brain and -retinal barriers and binds to Aβ fibrils and oligomers with high affinity [490, 527, 538–551], with the ability for *ex vivo* and *in vivo* visualization when specifically bound to retinal Aβ plaques (Fig. 7A, B) [485, 490, 527]. This approach enabled non-invasive detection and monitoring of desecrate retinal Aβ deposits in live animal models of AD [490], including the capability to track the dynamic appearance and clearance of individual plaques and their substantial reduction after glatiramer acetate immunotherapy [527, 552, 553].

In a proof-of-concept clinical trial, the safety and feasibility to non-invasively detect and quantify retinal amyloid deposits in live human patients was demonstrated using a modified scanning laser ophthalmoscope and a proprietary oral curcumin formulation (Longvida®) with increased bioavailability (Fig. 7I-M) [485]. Corresponding to the pattern reported in histological examinations, retinal amyloid deposits in living AD patients were frequently concentrated in the mid- and far-periphery of the superior hemisphere (Fig. 7K). A significant 2.1-fold increase in retinal amyloid index, a quantitative measure developed to assess numerical value of amyloid burden in the retina of living patients, was revealed in AD patients versus matched controls (Fig. 7L, M) [485]. Recent studies applying non-invasive retinal imaging in live AD patients, which detected NFL thinning [466, 477], increased inclusion bodies [554, 555], reduced blood flow, microvasculature alterations, and oxygen saturation in arterioles and venules [479, 556, 557], and importantly, hallmark Aβ deposits [485], are encouraging first steps toward the development of practical tools for predicting disease risk and progression. Since the retina in other ND such as multiple sclerosis, ischemic stroke, and Parkinson's disease also exhibits pathophysiological processes similar to those detected in the brain [501, 558–561], retinal imaging may also facilitate differential diagnosis for different proteinopathies, neurodegenerative and neurological diseases.

As research exploring AD in the brain, the possibility that the easily accessible retina may faithfully reflect AD neuropathology warrants further inves-

tigation. The preliminary evidence of retinal Aβ accumulation in early-stage cases together with the indication of amyloid-related neurodegeneration in the AD retina [466, 485, 490] suggests that AD is both a cerebral and an ocular disease, and may support retinal imaging as a screening tool even during the asymptomatic at risk stage. Future studies are needed to assess the nature of the relationship between cerebral and retinal amyloid burden in larger cohorts and in specific anatomical regions, and perhaps also to determine the potential link among cerebral amyloid angiopathy and retinal vascular amyloid. Given that retinal amyloid pathology could foretell brain disease and cognitive decline, it may prove essential for early detection of AD, predicting disease progression, and monitoring response to therapy.

In addition, non-invasive functional tests of pupil reactivity to light may complement the characterization of retinal abnormalities with imaging techniques [562]. Indeed, pupil responses to light stimulations are abnormal in AD patients [563], who show hypersensitive pupil-dilation to tropicamide, an acetylcholine receptor antagonist, as well as a diminished pupil light reflex [564, 565]. Although the retinal abnormalities mentioned above could account for these pupillary effects, the Edinger-Westphal nucleus, a major relay involved in pupil control where early signs of AD (cell loss and amyloid plaques) have also been observed, could also contribute to pupillary abnormalities. Conducting focal tests in different regions of the visual field to probe the pupil response can help identifying the functional consequences of the retinal amyloid imaging results. If the results of retinal imaging and functional tests were strongly correlated, pupil reactivity could be used as a proxy for AD severity, with the advantage that functional tests of pupil reactivity are easy, cheap and fast to perform, do not require a strong involvement of the patients, and can routinely be conducted to detect and track the evolution of AD, as well as the response to therapy.

In this regard, the "VISION" pilot translational neuroscience research program, belonging to the previously mentioned Sorbonne Université GRC-APM (GRC n° 21), has been developed and launched in an early asymptomatic preclinical population to assess retinal amyloid imaging for 1) screening of amyloid and tracking its progression as well as 2) predicting pathophysiological disease progression, cognitive decline, and conversion to prodromal AD. The non-invasive nature, easy accessibility and generalizability are appealing features regarding a potential context of use.

SPATIOTEMPORAL MODELING OF MULTIMODAL LONGITUDINAL DATA ANALYSIS

Nowadays, deepening our understanding of AD pathophysiology is made possible by the following biomarkers that can be derived *in vivo* from the subject: "fluid" from blood (e.g., genetic risk factors) and CSF (e.g., abnormal $A\beta_{42}$ and p-tau dosing); "structural" (e.g., brain atrophy as a sign of neurodegeneration) and "functional" (e.g., brain disconnection syndrome) from MRI, "molecular" (e.g., brain hypometabolism and deposition of $A\beta_{42}$ and p-tau) from PET, and "neurophysiological" (e.g., abnormal cortical neural synchronization and coupling). Furthermore, fine neuropsychological and clinical scales allow a detailed measurement of cognitive impairment, self-care, independence in living in a community, and mental disorders (e.g., anxiety, mood, psychosis, and behavior). All these measurements allow a personalized evaluation of cerebral residual capacity and function over time by the repetition of the recording sessions.

Keeping in mind this premise, a major issue is the identification of the best statistical and mathematical procedures, from computational neurosciences, weighting the information value of the above biomarkers and clinical indices for early diagnosis (even in preclinical or prodromal stages preceding dementia), monitoring, therapy response, and prediction of the disease evolution.

To this aim, digital brain models have been developed in recent years, as a way to synthetize a 3D geometrical model summarizing the anatomical invariants in a group of subjects [566–569]. This model has been extended recently to functional data [570, 571]. The main interest of such models is that they do not only illustrate the effects of the AD on brain structure and function at the group level but also include information about individual variability allowing the computation of the difference between a given patient and the reference groups of healthy subjects and patients with other dementing disorders to provide diagnostic information as sensitivity (detection of AD patients), specificity (detection of healthy subjects or patients with other diseases), and global classification accuracy.

These diagnostic models are based on the Bayesian inference of *non-linear* mixed-effects models, which complement the usual *linear* mixed-effects models typically used in biostatistics [569, 572]. This combination of statistical and geometric approaches

accounts for the inherent structure in the data such as the specific organization of the brain anatomy as prior knowledge. It allows the rendering of the inter-individual variability as a realistic and interpretable change of the 3D model. Individual characteristics are summarized by a multivariate descriptor, which may be used in turn to explore the distribution of the individuals in different clusters, to correlate it with external factors, or to use as input in machine learning algorithms to make individual predictions [568].

Ideally, such a static model should be adapted to account for the disease progression over time and provide prognosis of clinical evolution in individual AD patients. Digital models of brain ageing are constructed as dynamical models showing the complex spatiotemporal patterns of changes in the above biomarkers while the disease progresses. Inter-individual variability is expressed in terms of changes in individual spatiotemporal trajectories. The construction of such models of disease progression results from several key components [570, 571, 573–576]: 1) artificial intelligence approaches that are used to combine several short-term data sequences in longitudinal data sets to synthetize a long-term scenario of disease progression; 2) different data modalities that are integrated in the model by converting them into a common abstract mathematical space (called a Riemannian manifold) where statistical distributions of spatiotemporal trajectories may be rigorously defined; 3) variability in trajectories accounting for the direction of the trajectories and the dynamics at which these trajectories are followed.

Each individual disease trajectory is now positioned in a spatiotemporal coordinate system, where a multivariate descriptor encodes the variability in the direction of the trajectory, and dynamical parameters encode for the variability in age at disease onset and pace of disease progression. Given the observation of a new subject at one or few time-points, one may personalize the scenario of disease progression by adjusting model parameters, thus transferring the knowledge gained from the automatic analysis of a longitudinal data set to this new individual. This personalized model may be utilized then to predict the future state of the subject, for instance the time to the onset of a specific symptom. We have employed such an approach to predict the time-to-diagnosis in mild cognitive impaired subjects using a model of cognitive decline from neuropsychological assessments [577], and to predict the future map of cortical thickness for the same subjects using structural imaging [571]. This approach opens up the way to build efficient decision support systems for monitoring disease progression and selecting patients in clinical trials with a specific biomarker-based diagnosis of AD, at a specific disease stage (e.g., preclinical, prodromal, or manifest dementia) and with an expected pattern of progression.

In addition, such a personalized scenario may offer a new way to assess treatment efficacy by evaluating to which extend it changes the disease trajectory, that is the complex non-linear spatiotemporal patterns of changes. This approach evolves the standard procedure based on annual percentage rate of an outcome measure since: 1) it does not assume a linear variation of the outcome at all disease stage but account for the non-linear dynamics of changes across disease stages, and 2) it makes use of a multivariate descriptor of disease trajectory and not only a univariate outcome measure.

THE EMERGING FIELD OF SYSTEMS PHARMACOLOGY IN ALZHEIMER'S DISEASE

The consequences of the highly complexity of AD pathophysiology can be clearly observed in the results of drug development pipeline for the disease: out of 413 clinical trials conducted during the 2002 to 2012 period, 99.6% failed [578]. Moreover, a review of AD drug development pipeline in 2016 showed that although the pipeline has increased in size, it is significantly smaller compared to the cancer field, and that the most common target (76%) is still amyloid, reflecting the urgent need for deeper understanding the pathophysiology of the disease [579]. In fact, disappointing results of anti-amyloid drug candidates can be attributed to three major factors relating to drug discovery and development, namely 1) inter-species mechanistic differences between animal models and human, 2) complex biology of Aβ in relation to disease staging, and 3) ignorance of non-amyloid pathways. Thus, it is imperative to delineate the complexity of AD pathophysiology using systems biology-based approaches, which take advantage of computational analysis and modeling of both quantitative (e.g., "omics"-based) and qualitative (e.g., literature-based) data. The goal of systems biology methods is to aid researchers develop hypotheses regarding the disease system and gain better mechanistic insights into the

pathophysiology and progression of disease across multiple biological scales and time. Mechanistic systems models are either mathematical representations of pathophysiologic processes or computable cellular networks but the latter has gained more attention for analysis of drug action [580]. Since these models use networks instead of single transduction pathways, complex patterns of drug action within the target biological context can be studied in more details, a field that has emerged as systems pharmacology.

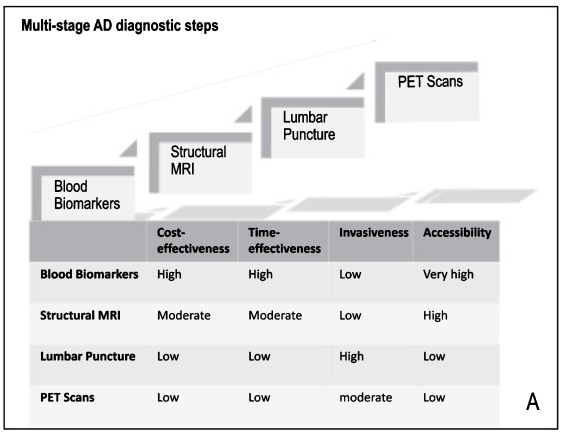
According to the American Association of Pharmaceutical Scientists (AAPS), systems pharmacology is "*the science of advancing knowledge about drug action at the molecular, cellular, tissue, organ, organism, and population levels*" (available at http://www.aaps.org/Systems_Pharmacology/). To obtain full understanding of drug action at the systems level, we need to combine disease mechanism, pharmacodynamics, and pharmacokinetic data into a single model. However, incorporation of quantitative parameters and measurements increases the model complexity so that special mathematical techniques are required to reduce the number of parameters without affecting the behavior of the system; thus, disease mechanistic models are considered as the first substrate for building full-fledged systems pharmacology models [581]. Disease mechanistic models are molecular and cellular networks that aim to elucidate the impact of therapeutics or new drug candidates on impaired biological functions under disease conditions. The key to usefulness of disease models is context-sensitivity, meaning that disease network models should represent the real-world context in terms of cell and tissue type (spatial dimension), disease sub-type (functional dimension), and progression stages (temporal dimension). It is only in the right context that correct inferences, interpretations, and predictions can be made out of the model. The focus of earlier models was to relate drugs to proteins in the form of drug-target networks where protein-protein interaction networks were used as the fundamental model for interpretation of drug mode-of-action [582]. Interestingly, these models also revealed an important aspect of systems pharmacology paradigm, which was conceptualized and coined as "polypharmacology" [583]. This concept changed the single-target approach to designing new drugs in the discovery phase because topological analysis of drug targets in network models demonstrated that a compound binds to multiple targets. As a consequence, a drug hits additional targets, known as off-targets, which leads to side effects. Campillos and colleagues (2008) used drug-drug and drug-target networks enriched with side-effect phenotype information for all approved drugs across many disease indications and based on side-effect similarities predicted and experimentally validated novel drug-target relations [584]. This approach enables researchers to predict off-targets and thereby probable side effects for candidate drugs in preclinical settings. The so-called structural systems pharmacology aims at modeling energetic and dynamic modifications of genomic macromolecules including proteins, DNA, and RNA by drug candidates [585]. This strategy has been implemented by Nikolic and colleagues (2016) to predict both primary target and off-target profiles of several anti-neurodegenerative compounds based on their chemical structures [586]. Their analysis resulted in identification of novel compounds that hit multiple targets and inhibited acetylcholinesterase, butyrylcholinesterase, monoamine oxidases A and B in the context of AD pathophysiology. Moreover, knowing which drug properties distinguishes CNS drugs from others can help drug designers select those properties in the new drug candidates that confer the least side effects and the best efficacy. To this end, Shahid and colleagues (2013) developed a computational method that identified and classified neurodegenerative drugs from non-neurodegenerative drugs with 80% accuracy [587]. DrugGenEx-Net is a computational platform that predicts disease-specific drug polypharmacology based on multi-tiered network analysis of drug-target, disease-target, pathway-target and target-target interactions [588]; the model revealed that Sunitinib, an approved drug for renal cell carcinoma, hits multiple targets associated with AD pathways and thus can be considered for repurposing.

With advancements in systems biology modeling languages, such as Systems Biology Markup Language (SBML) and Open Biological Expression Language (OpenBEL), drug-mode-of-action can now be investigated in a context-sensitive, rich environment that goes beyond simple representation of protein-protein interactions by including various types of biological entities covering genotype to phenotype scales. For instance, Fujita and colleagues (2014) developed a comprehensive molecular interaction map of Parkinson's disease that included major signaling pathways in Parkinson's disease, modeled and presented in SBML format; however, they did not include drug information

in their model [589]. AlzPathway is the result of an early initiative that attempted to systematically collect AD-related signaling pathways from literature and bring them together within the first map of cellular AD signaling pathways, represented in SBML [590]. Recently, Iyappan and colleagues (2016) identified all signaling pathways reportedly involved in the human ND, mapped them back onto their corresponding anatomic sites on the human brain, and used these pathways for explaining the mode-of-action of the AD approved drug, Rasagiline [591].

In the past years, with the availability of increasing amount of data and knowledge on the one hand, and emergence of new computational biology methods on the other, the IDM framework has increasingly drawn more attention by academic and pharmaceutical research groups. The models generated by this approach combine data-driven and knowledge-driven models into a single integrative model and represent signaling pathways with cause and effect relations [23]. However, a major challenge for this approach is integration of heterogeneous datasets and information that come from various data sources. For instance, the ADNI provides big neuroimaging data along with genetic and biomarker data from AD and MCI subjects [592]. If integrated into predictive models, ADNI data will have maximal impact on the AD drug research. But, the first step toward IDM is standardization and harmonization of different datasets so that they are semantically compatible. Ontologies are semantic frameworks that provide a reference for standardization and harmonization of diverse datasets. For instance, AD ontology (ADO) has been developed to provide such a reference for AD knowledge domain [593]. ADO was used by Kodamullil and colleagues (2015) to represent scientific findings in a computable, cause-and-effect model of AD pathology, which was designed and coded in Open Biological Expression Language (available at http://openbel.org/) [594]. This model contains causal and correlative relationships between biomolecules, pathways, and clinical readouts and was used for model-guided interpretation of genetic variation data for a comorbidity analysis between AD and type 2 diabetes mellitus. Similarly, drug-target interactions and drug mode-of-action can be investigated and predicted using these models. Indeed, integrative models that encompass data from genome to phenome across biological scales from cells to clinical outcomes, enable us to predict the mode-of-action of candidate drugs within the right pathophysiological context and in a multidimensional space of human biology. Perhaps one of the most fundamental works in this area is the study by Emon and colleagues (2017) who systematically analyzed the brain chemical space and identified drug candidates for repositioning in AD [595]. They first generated a large model in BEL containing genes, proteins, drugs and chemicals, biological processes, and disease concepts in the context of neurodegeneration. Then, by mechanistic analysis of this model, they not only suggested Donepezil as repurposing candidate for amyotrophic lateral sclerosis, but also found a mechanism of action by which Riluzole, a drug used in amyotrophic lateral sclerosis, could be predicted to interfere with several pathophysiological pathways in AD. Moreover, the mode-of-action analysis of other drugs in the context of AD using this model predicted that Cyclosporine, a drug used for treatment of rheumatoid arthritis, which shares common targets with 5 approved drugs for AD, can exert neuroprotective effects. Several lines of evidence that experimentally proved its anti-AD effects supported this prediction.

Currently, several initiatives have undertaken the effort to facilitate systems pharmacology studies in the field of ND in general and AD in particular. The AETIONOMY project, funded by the Innovative Medicine Initiative (see http://www.imi.europa.eu/), has already set up a specialized knowledgebase for ND with focus on AD and Parkinson's diseases, and takes an integrative modeling approach to computationally predict and clinically validate mechanistic signatures that stratify AD and Parkinson's patients (see http://www.aetionomy.eu/). The mission of this project is to lay foundation for development of new drugs targeting patient subgroups and thus promoting personalized medicine. The Brain Health Modeling Initiative (BHMI) is another project that takes advantage of integrative mechanism-based computational models and simulations using big data with the aim of matching right targets and biomarkers for optimal drug design in AD [596]. The European commission-funded project SysPharmAD proposes a systems pharmacology approach to the discovery of novel therapeutics in AD using an integrative network model that combines "omics" data with stage-specific clinical data. The aim of this project is to design and validate a systems pharmacology strategy based on AD staging that helps researchers identify synergistic multi-targeting compounds modifying the disease path (available at http://cordis.europa.eu/project/rcn/185567_en.html).



	Cost-effectiveness	Time-effectiveness	Invasiveness	Accessibility
Blood Biomarkers	High	High	Low	Very high
Structural MRI	Moderate	Moderate	Low	High
Lumbar Puncture	Low	Low	High	Low
PET Scans	Low	Low	moderate	Low

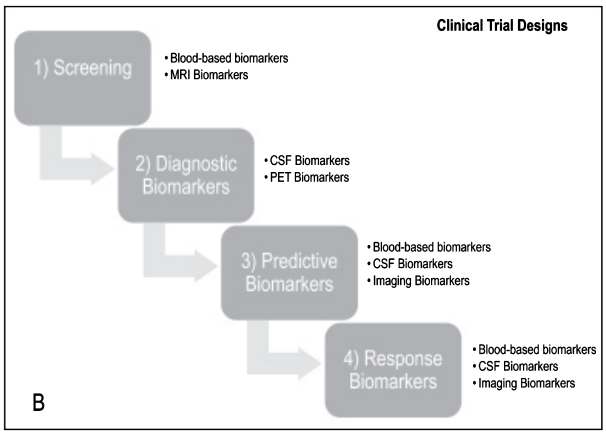


Fig. 8. Evolving spectrum of biomarkers and modalities. A) The ideal biomarker should be minimally-invasive, unexpansive, practical, rapid and reliable with low level of expertise required. Therefore, in the clinical-setting, biomarkers should be assessed in a multi-stage diagnostic workout carried-out along four steps (blood biomarkers, structural MRI, lumbar puncture, PET scans) according to the overall balance among the following factors: cost-effectiveness, time-effectiveness, invasiveness and accessibility. B) Biomarkers represent one strategy to tailor therapy. The idealistic markers for ND would enable their implementation in screening, diagnosis, progression of the disease, and monitoring of the response to therapy. Therefore, in clinical trials, biomarkers can be used for several purposes: 1) to identify people eligible for the trial, i.e., those considered at high risk for ND (screening biomarkers); 2) to guide clinical diagnosis (diagnostic markers); 3) to optimize treatment decisions, providing information on the likelihood of response to a given drug (predictive biomarkers); 4) to detect and quantify the response rate to treatment (response markers). MRI, magnetic resonance imaging; PET, positron emission tomography; ND, neurodegenerative diseases.

CONCLUSIONS

The multidimensional nature of all ND, AD included, is well established to-date, along with the fact that their onset and progression arise from dysregulation processes which evolve at both intracellular and extracellular levels. At the cellular level, ND are characterized by dystrophic neuronal structural changes leading to loss of function and, eventually, cell death. These phenomena spread in a "cell-to-cell" fashion in which intraneuronal protein misfolding affects structural plasticity in a nearby neuron by self-propagation of pathogenic protein aggregates. This, in turn, leads to decreased dendritic spines and synaptic sites density, and, eventually, loss of brain connections.

At the subcellular and molecular level, the core pathophysiological phenomenon is represented by failure of proteostasis cellular pathways [597, 598], from protein misfolding and aggregation to decreased clearance, mitochondrial dysfunction, loss of cell homeostasis, and, consequently, enhanced cell signaling pathways related to apoptosis. Therefore, ND are initially characterized by several alterations of subcellular frameworks, mostly concerning proteostasis, on which both the anatomy and physiology of neurons and glial cells are founded.

The genome, through mutual interactions with endogenous and exogenous factors, leads to a wide spectrum of variations at the level of proteome and metabolome that, incontrovertibly, account for both intracellular and extracellular integrity. As a result, the systems biology and systems neurophysiology paradigms can provide a conceptual model where structural and functional networks are dynamically interconnected across different dimensional levels into accounting a multiscale dynamical system which has already been seen to manifest also into peripheral branches like the autonomic nervous system in health and disease [599, 600].

At present, there is an urgent need to identify a large array of reliable biomarkers to *in vivo* identify the above mentioned interacting multidimensional levels which characterize ND. Such biomarkers need to be able to chart the spatio-temporal trajectories of complex brain pathophysiological mechanisms, at the same time taking into account interindividual variables. Complex, time varying higher order statistics as well as structural model should also be considered within the systems neurophysiology modeling approach [601–604]. Pathophysiological biomarkers are required to track the pathophysiological mechanisms underlying ND (Fig. 8). For instance, cerebral amyloid-PET is commonly considered as a

molecular proxy of the Aβ metabolism impairment rather than a conventional biomarker of neocortical deposition of neuritis plaques. In this context, biomarkers are the appropriate tools for developing receptor-tailored drugs, as already demonstrated and currently practiced in the field of oncology. Both structural and functional brain markers are expected to elucidate the link between clinical phenotypes and molecular pathophysiological mechanisms.

Notably, cerebral ^{18}F-FDG-PET is commonly used as prognostic indicator in several clinical trials on AD and other ND. Indeed, the early recovery of specific brain functions or networks is crucial to identify downstream effects of disease therapies, even before measuring the clinical benefit. As another example, in the context of identifying brain biomarkers from non-invasive imaging within a more individually tailored, PM-based approach, recent developments have pointed out the concept and added value of "dense sampling of individual brains" [605–607]. This interesting development is based on the realization that, while a large body of research is accustomed to averaging neuroimaging data across individuals and, hence, implicitly assuming a high degree of functional homology, by definition there must be a finer scale at which this homology breaks down, possibly the scale which encodes the individual idiosyncrasies at the base of a unique individual's disease trajectory and/or therapy response. By sampling relatively few brains for several hours, the authors demonstrate how individual differences in well-known networks, e.g., the default mode and the salience network, are clearly visible. Therefore, it is possible that future developments in neuroimaging will shift more toward longer (several hours/days) sampling of individual brains/patients, thus providing more solid bases for the implementation of the "precision neuroscience" paradigm that will likely be needed to understand ND.

Interestingly, functional and topographic biomarkers could also be employed in identifying the adequate target. In particular, they could be valuable in detecting specific brain areas for potential trials of targeted neuromodulation, thus providing comprehensive information on regional atrophy, impaired connectivity, metabolic alterations, and regional decrease of cerebral blood flow. Finally, both clinical examination and full psychometric evaluation still remain the first-line approach in identifying pathological phenotypes supporting the whole diagnostic workout. For instance, to date, the identification of hippocampal-like amnestic impairment supports the clinical diagnosis of AD, thus justifying an anticholinesterase inhibitor-based treatment. Notably, in the context of a systems biology- and systems neurophysiology-based interpretation of ND phenotype, clinical markers should be considered the highest level "descriptors" of the disease and represent the ultimate measures to identify effective therapies.

In summary, the future implementation of the systems biology and systems neurophysiology paradigms, based on the integrated analysis of big and deep heterogeneous data sources, will be crucial to reach a deeper understanding of the pathophysiology of AD and other ND. The main challenges ahead will certainly lie in the development of analytical applications capable of processing massive quantities of stored laboratory and clinical data. Against this backdrop, the big data approach should be leveraged to maximize the information that can be extracted from preclinical and clinical records, ultimately augmenting our knowledge regarding the molecular, cellular, and systems processes underlying AD development. As we unravel the dynamic and longitudinal changes of the biomarker landscape in AD, we will make a further step toward a holistic understanding of the natural course of the disease. Integrating different sources of information will enable researchers to obtain a new integrated picture of the pathophysiological process of the disease that will span from molecular alterations to cognitive manifestations. In this scenario, the Big Data Research and Development Initiative (available at https://obamawhitehouse.archives.gov/blog/2012/03/29/big-data-big-deal), promoted by the previous Obama Administration under the "Big Data is a Big Deal" motto, is expected to accelerate progress toward a new era of PM in AD. This ultimate mission will be accomplished by assembling, linking, and harmonizing big data to facilitate high-impact, multidisciplinary, and collaborative research efforts. After a decade of failed clinical trials in AD, the adoption of "big data science" within an IDM theoretical framework by the international APMI allowed us to enter into a transformative research scenario. It is currently expected that PM will underpin most, if not all, of the prevention and treatment advances yet to come. Significant breakthroughs in our understanding of the early phases of AD and other ND and the rapid advent of new laboratory technologies are providing unprecedented opportunities to make a major impact on the natural history of AD at the earliest pre-

clinical asymptomatic stage [608]. We are currently standing at the edge of a new frontier that will thoroughly explore the molecular and cellular events that drive the development of the disease before cognitive symptoms are evident. New preventive approaches and therapies developed through PM may improve compliance and increased level of trust and confidence among all stakeholders and reduce the number of failures. In this context, we are expected to move swiftly from the traditional "one-size-fits-all – magic bullet therapies" scenario to a personalized PM-based approach. The unprecedented effort promoted by the APMI is ultimately tailored to implement a paradigm shift in AD research which will be backboned by large, international, and interdisciplinary collaborative academic, private and industry networks. The field of PM does not lack for enthusiastic, dedicated pioneers who are moving forward expeditiously to clinical adoption. As the evidence base supported by the APMI expands, much more can and should be done to accelerate the process for the benefit of individual patients, the healthcare system, and society overall.

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Multifactorial Hypothesis and Multi-Targets for Alzheimer's Disease

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Abstract. The amyloid cascade hypothesis has been dominating drug discovery for Alzheimer's disease (AD) for the last two decades. The failure of the development of effective drugs for slowing down or reversing the progression of AD warrants the AD field to consider out-of-the-box thinking and therapeutic approaches. We propose the multifactorial hypothesis of AD, emphasizing that AD is caused by multiple etiological factors, which may result in common brain pathology and functional consequences through several separate but integrated molecular pathways. More than one etiological factor and mechanistic pathway may be involved in a single individual with sporadic AD, and different individuals may have different etiological factors, involving different mechanisms/pathways. We urge the recognition of the multifactorial nature of AD and the paradigm shift of AD drug development from a single target to multiple targets, either with the multitarget-directed ligands approach or the cocktail therapy approach. We believe that patient stratification and the use of the precision medicine model will also benefit AD drug discovery.

Keywords: Alzheimer's disease, cocktail therapy, multifactorial hypothesis, multitarget-directed ligands, patient stratification, precision medicine model

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and is characterized by chronic, progressive neurodegeneration that leads to cognitive impairment and eventually to dementia. In familial, early onset AD, the disease is caused by certain mutations in the genes of presenilins or amyloid-β protein precursor (AβPP). Over 95% of AD cases are sporadic in nature and are not caused by any known gene mutations. Both familial and sporadic AD are characterized by two important brain lesions: aggregation of amyloid-β (Aβ) into amyloid plaques and of hyperphosphorylated microtubule-associated protein tau into neurofibrillary tangles. The presence of amyloid plaques, neurofibrillary tangles, and neuronal/synaptic loss in the brain are the characteristic histopathological hallmarks of AD.

The modern era of AD research at the molecular level began in 1980s. During the last three decades, many molecular pathways involved in or relevant to the mechanisms of AD have been learned. However, modern AD research has not yet led to the development of any drug that can slow down the progression of AD or cure the disease. Only one drug, memantine, was developed that is still symptomatic and has moderate efficacy in temporarily reducing symptoms for only moderate or severe AD [1].

The failure of developing good effective drugs for AD, despite enormous amounts of resources and effort invested in the last 2-3 decades, led the AD field to think seriously what we have done, where we

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stand now, and where we should head in AD research. This reconsideration is obviously seen in the last several years and has led many to doubt or even abandon the amyloid cascade hypothesis and shift their efforts to other possible mechanisms or targets, such as tau pathology, for their research and drug development [2–5]. At this transition time for AD research as well as the 110th year anniversary of the first publication of AD by Alois Alzheimer, it is especially timely and important to have a collection of ideas and opinions from AD experts, as organized by the *Journal of Alzheimer's Disease*, regarding the new beginnings of AD research.

The failure of AD clinical trials to date could result from many reasons, which have been discussed recently [6–8]. These reasons include the complex nature of the disease, limits of animal models for preclinical studies, inadequate designs of clinical trials, and many others. One important reason is probably the lack of appreciation and understanding of the multifactorial nature and mechanisms of the disease. To date, most AD clinical trials have been based on a single mechanism or pathway.

In addition to the dominant amyloid cascade hypothesis [9, 10], several other hypotheses have been proposed for the mechanisms of sporadic AD. These hypotheses include the cholinergic hypothesis [11, 12], tau hypothesis [13, 14], mitochondrial hypothesis [15, 16], oxidative stress hypothesis [17, 18], neuroinflammation hypothesis [19], brain insulin resistance hypothesis [20, 21], brain metabolic hypothesis [22–24], calcium hypothesis [25], innate immunity hypothesis [26, 27], and others. All these AD hypotheses are backed by substantial support from research data. This is actually not surprising because, as an age-associated neurodegenerative disease, many factors may initiate the development of AD and many molecular pathways may mediate the progression of the disease in the aged brain. However, a common problem of these hypotheses is that they intend to overemphasize the specific mechanism/pathway proposed and undervalue other mechanisms and heterogeneity. Such a narrow focus appears to attribute to the failure of AD drug development during the last decades.

Sporadic AD is caused by multiple etiological factors, which may result in common pathological brain damage and functional consequences through several separate but integrated molecular pathways. The multiple etiological factors and mechanistic pathways are likely involved in a single individual with sporadic AD, and different individuals may have different etiological factors and involve somewhat different mechanisms/pathways. This article discusses the multifactorial mechanism and multi-targets for AD.

THE MULTIFACTORIAL HYPOTHESIS OF AD

The development, growth, and maturation of a human body reaches its peak in the third decade of life. Human brain, as a special organ, may further mature for decades due to continuous learning and new experience. However, wearing and aging of the human brain starts at middle age. Normal aging is a constant balancing between physiological aging plus pathological risks/insults and the natural defense mechanisms (Fig. 1A). There are many risks and insults that occur and accumulate during aging, including genetic risks, epigenetic and metabolic factors, and environmental insults. The human body also responds to these factors/insults with its defense mechanisms, which could include general defense and those specific to individual insults. The balance between aging/insults and the defense mechanisms is dynamic and can shift within a certain range under physiological conditions. During normal aging, although the right side of the balance shown in Fig. 1A can be heavier as the accumulation of factors/insults, such as factor A to G, the balance tilts to the right side but still maintains within the normal range. However, as one or more of these factors/insults get heavier or new factors/insults (e.g., factor H, I, etc.) are added up, the imbalance eventually reaches the threshold and breaks the balance, i.e., initiation of the development of AD. These factors/insults collectively result in neurodegeneration, leading to cognitive impairment and eventually dementia, through individual molecular pathways (Fig. 1B). Some of these pathways involve in Aβ overproduction/aggregation and tau hyperphosphorylation/aggregation, leading the formation of amyloid plaques and neurofibrillary tangles as the two hallmark brain lesions of AD.

Our proposed multifactorial hypothesis can perfectly explain why aging is the most important risk factor for AD, as the defense mechanisms on the left side of the balance shown in Fig. 1A becomes weaker during aging. On the other side, healthy lifestyle, such as physical and intellectual exercises and healthy diet, can help the defense mechanisms and thus inhibit or delay the onset of the disease.

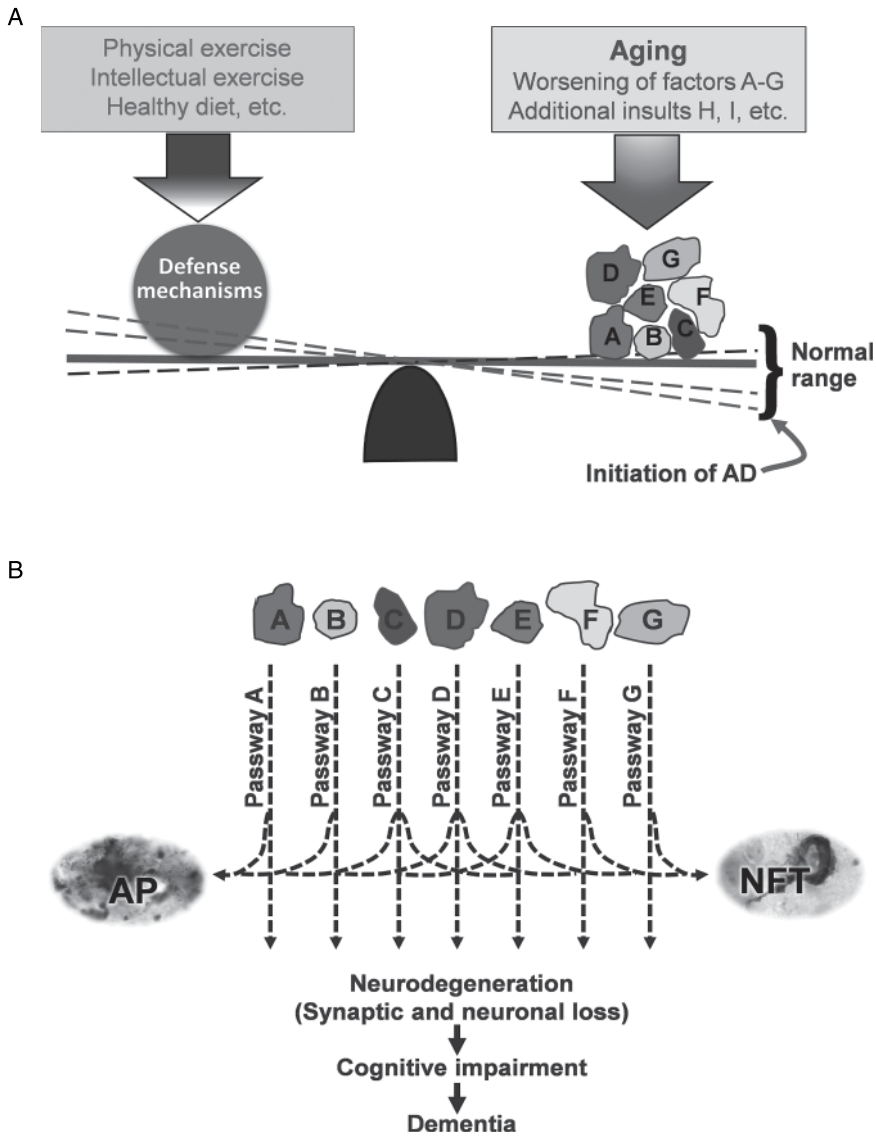

Fig. 1. The proposed multifactorial hypothesis of AD. A) The balance between the potential factors/insults accumulated during normal aging and the defense mechanisms. Worsening of these factors/insults (such as A to G) and/or adding of additional insults (such as H, I, etc.) can initiate the onset of AD. B) The multifactorial insults collectively cause neurodegeneration through multiple molecular mechanisms/pathways and consequently cognitive impairment and dementia. Some of these pathways also lead to the formation of amyloid plaques (AP) and neurofibrillary tangles (NFT), which are part of the end products of these pathways and also hallmark brain lesions of AD.

Multifactorial mechanisms of AD have been proposed previously, which state that more than one etiopathological factors and mechanisms are involved in the pathogenesis of AD [28–30]. However, the multifactorial hypothesis of AD that we proposed here is different from those proposed previously. Our hypothesis emphasizes two key concepts for the development of AD. First, we emphasize that the development and onset of sporadic AD result from the *collective* effects of multiple factors/insults that are not restricted to one or more specific insults. This emphasis warrants targeting more than one insults/pathways simultaneously for effective AD therapy. Second, we emphasize that each individual may have a different combination of etiological factors/insults that cause the onset of AD in this particular individual. This emphasis recognizes the diversity of etiological factors and molecular mechanisms among individual AD cases and justifies the stratification of AD patients and the use of precision

medicine concept for the treatment of AD, which will be discussed below.

The previously proposed hypotheses of AD, such as the amyloid cascade hypothesis, tau hypothesis, and neuroinflammation hypothesis, are all supported by more or less experimental and clinical evidence. Our proposed multifactorial hypothesis does not conflict with those hypotheses but include each of them as one factor/insult for the disease development. Instead, we believe that we need to consider all parts of the issues of AD simultaneously when designing and testing new AD therapeutics. One major problem with previously proposed hypotheses is to emphasize one pathway but overlook or even ignore all others. This problem, in our opinion, partially accounts for the failure of all AD drug development so far.

Each factor/insult of the right side of the balance of Fig. 1 may have different weights and contribute differently to the initiation and development of sporadic AD, and in different individuals the weight of each factor/insult may be different. Extensive research accumulated during the last three decades suggest many important factors/insults. They include aging, as well as genetic, epigenetic, metabolic, and environmental factors. Some of these factors/insults, such as mutations of presenilins and AβPP, appear to be so strong that they can lead to early onset familial AD without co-existing of other insults. However, in most AD cases, amyloid or tau pathology is insufficient to lead to sporadic AD, as these pathologies can also be seen in the brains of individuals without cognitive impairment.

STRATEGY TO TARGET THE MULTIFACTORIAL MECHANISM OF AD

An overview of the AD clinical trial data indicated that, over the last decade, more than 50 drug candidates have successfully passed phase II clinical trials, but none has passed phase III [31]. According to our proposed multifactorial AD hypothesis, it is not surprising that all the clinical trials targeting to a single pathway or mechanism critical to AD have failed so far. This is obvious because sporadic AD is caused by an imbalance of a *collective* action of several insults, as shown in Fig. 1. Inhibiting or removing only one of them is unlikely to be sufficient to restore the balance to the normal range. It is time for us to take a paradigm shift for AD drug development to a multi-targets approach on the basis of the multifactorial AD hypothesis (Fig. 2).

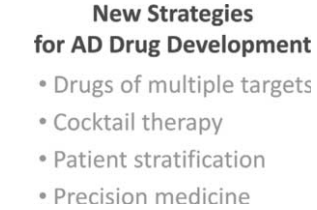

Fig. 2. Proposed strategies for AD drug development on the basis of the multifactorial hypothesis.

It is generally much more difficult to design a drug that can act at multiple targets. However, this is not impossible. A few groups in Europe and China have started the approach of multitarget-directed ligands (MTDL) for AD drug development [32–36]. The aim of MTDL design is to combine features that can interact with two or more of the desired targets. The MTDL molecules can be conceived to directly interact with multiple targets associated with AD by the molecular hybridization of different pharmacophore moieties from already identified bioactive molecules [37, 38]. Each pharmacophore of the new hybrid drug can preserve the capacity of interacting with their specific sites on the targets and thus generate multiple specific pharmacological responses, which would enable the treatment of multifactorial AD. The development of MTDLs can prevent the challenge of simultaneously administering multiple drugs with potentially different degrees of bioavailability, pharmacokinetics, and metabolism. Thus, this pharmacological approach can also provide patients with a simplification of the therapeutic regimen.

Another approach, which is probably more practical, is to select substances of multiple actions against various insults/mechanisms involved in AD from nature sources. There are several natural compounds, such as isaindigotone, chelerythrine, chalcone, coumarin, huprine, curcumin, rhein, berberine, and resveratrol derivatives, that deserve investigation for AD drug development. This approach may indicate new directions for the development of new anti-AD drugs.

The third strategy is to consider simultaneous treatments with more than one drugs targeting various insults/mechanisms according to the multifactorial AD hypothesis. Such an approach had been used effectively in chemotherapy and in fighting against HIV/AIDS as the cocktail therapy. The cocktail therapy is proved to be essential to such an infective disease with a clear single cause, infection with the HIV virus. It actually makes more sense to employ such an approach for fighting

against AD, a disease with multiple etiologies and mechanisms.

Another important strategy for AD drug discovery is to stratify AD patients based on their likely factors/insults and test AD drug candidates in the stratified population of AD patients. Because the sporadic AD can be caused by a combination of various etiological factors and different molecular mechanisms/pathways may dominate in different populations, AD can be categorized into different subgroups, and different subgroups likely represent different etiopathogenic mechanisms and possibly also somewhat different clinical profiles. On the basis of the levels of tau, ubiquitin, and $A\beta_{1-42}$ in the cerebrospinal fluid, we were able to stratify AD patients into at least five subgroups [39]. Importantly, each of these five subgroups presented a different clinical profile. A recent study demonstrated structural variation in Aβ fibrils from AD clinical subtypes [40], suggesting some molecular and structural basis for AD subgroups. Therefore, testing a specific drug candidate in the stratified subgroup of AD patients, rather than the mixed populations of all AD patients, will certainly increase the likelihood of success in clinical trials. With the latest advances of brain imaging techniques and AD biomarkers, stratification of AD cases is now already feasible and will soon become more practical.

Precision medicine is a new medical model that proposes the customization of medical treatment and care to the individual patients. This model has been used successful for treating certain cancers [41]. In light of the unsuccessful investment of vast amount of effort and resources for AD drug discovery in the last two decades, it is time to make a paradigm shift and consider the precision medicine model for AD drug discovery and for future management of AD patients. Our knowledge of the disease-causing mutations of *PSEN1, PSEN2*, and *APP* for familial AD and of *ApoE* alleles and polymorphisms of some genes, such as *TREM2*, as risk factors for sporadic AD already make the use of precision medicine model for treating AD possible. Brain imaging and biomarker data can add additional values for customization of individual AD patients.

MULTI-TARGETS FOR TREATING AD: CURRENT STATUS

The multifactorial nature of AD means that there are many potential therapeutic targets. Targeting these targets individually with current drugs has been ineffective for AD in clinical trials. A possible answer lies in a polypharmacological approach to modify activities of several of these targets simultaneously, especially those associated with the pathogenesis of the disease. The main therapeutic targets currently under investigation for treating AD include key proteins (Aβ and tau) and their processing, receptors (cholinergic, glutamatergic, serotoninergic, dopaminergic, noradrenergic, histaminergic), enzymes (cholinesterase [ChE], α-, β- and γ-secretase, monoamine oxidases [MAO], O-GlcNAcase), and pathways/processes (insulin signaling, excitotoxicity, neuroinflammation, oxidative stress, neurogenesis, calcium and metal homeostasis, endoplasmic reticulum, and mitochondrial damage), all of which have been shown to be involved in the pathogenesis of AD.

Initial efforts on the multi-target strategy for treating AD are mainly focused on the development of compounds that have ChE inhibitor activity (tacrine- and donepezil-related derivatives) plus one or more properties of anti-Aβ aggregation, β-secretase inhibition, promotion of non-amyloidogenic cleavage of AβPP, MAO inhibition, neuroprotection, anti-oxidation, metal-chelating, NMDA (N-Methyl-D-aspartate) antagonist, nitric oxide-releasing, anti-inflammatory, tau hyperphosphorylation inhibition, and binding to serotonin receptors or opioid sigma 1 receptors. Tacrine is among the most popular pharmacophores used for the design of MTDLs since it is very active cholinesterase inhibitor. There is also a number of hybrid compounds containing fragments of donepezil, galantamine, or memantine. The pharmacologies and initial evaluations of these compounds have been recently reviewed by Guzior et al. [42] and Ismaili et al. [43] and thus are not discussed here in detail.

Examples of these hybrid compounds under investigation comprise the dual binding site of ChE inhibitors with additional properties such as anti-Aβ aggregating activity [44, 45], neuroprotective and antioxidant activity [46, 47], calcium channel blocking [48, 49], cannabinoid CB1 receptor antagonism [50], BACE-1 inhibition [51, 52], histamine H3 receptor antagonism [53], NMDA receptor channel blocking [54], serotonin 5-HT3 receptor antagonism [55], or serotonin transporter inhibition [56]. Other examples of dual-acting ligands are MAO-B inhibitors with iron-chelating agents [57], metal chelators with BACE-1 inhibitors [58], metal chelators with antioxidants [59], and modulators of

γ-secretase with PPARγ activities [60]. Most of these multifunctional ligands have been shown to display biological activity *in vitro* and require verification in animal models. However, several compounds like bis(7)-tacrine [61], ladostigil [62, 63] and memoquin [64] showed promising activity *in vivo* and in preclinical or even clinical studies.

Several groups have synthesized and assessed compounds bearing the N-benzylpiperidine group present in donepezil and the N-propargylamine motif present in PF9601N, a potent and selective MAO-B inhibitor with neuroprotective activities *in vitro* and *in vivo* [34]. Both scaffolds were linked by different heterocyclic ring systems, such as pyridine, indole or 8-hydroxyquinoline, allowing facile synthesis of different MTDL molecules for AD therapy. In addition to inhibiting ChE and MAO, some of these new MTDL molecules also have antioxidant, anti-Aβ-aggregating, anti-inflammatory, anti-apoptotic, and metal-chelating properties. Preclinical studies suggest that these MTDL compounds can target the multiple pathways involved in the pathogenesis of AD and thus represent a potential improvement of the current pharmacological therapy of AD. One example of MTDL model that progressed to clinical trials against AD is ladostigil, designed to inhibit MAO and ChE but also incorporating potent anti-apoptotic and neuroprotective activities [63]. The MTDL attempt combining activities of MAO and ChE has been reviewed recently [65].

The use of the well-known AD drugs donepezil, tacrine, or rivastigmine [47, 66] and bioactive natural products such as curcumin [67], berberine [68, 69], or 8-hydroxyquinoline [70]; as structural scaffolds for the development and search of new chemical entities with multiple properties for the treatment of AD has been investigated. These new hybrid compounds should be considered as simplified versions or lead drugs possessing potential as real alternatives to the current unsuccessful drugs for treating AD.

Another approach for the multi-target AD drug development is repurposing, i.e., the development of existing or abandoned drugs for new indications, related to the original purpose or after off-target effects are identified by data mining. Repurposing can reduce the time to launch, cost of development, and the uncertainty associated with safety and pharmacokinetics. Data mining is a way of using pre-existing knowledge about molecules and applying it to develop new drugs [71]. The most promising drug currently being investigated for repurposing is rasagiline, a selective, irreversible MAO-B inhibitor for the treatment of Parkinson's disease. The repurposing for AD was due to its ability to regulate the non-amyloidogenic processing of AβPP [72]. Rasagiline also has a neuroprotective activity due to the propargylamine moiety that activates Bcl-2 and downregulates the Bax proteins [73]. One phase II trial of rasagiline sponsored by Teva Pharmaceutical Industries was completed without publication of the results, and another phase II trial sponsored by the Cleveland Clinic is undergoing (https://www.clinicaltrials.gov/ct/show/NCT02359552).

Another example of repurposing for AD treatment is anti-diabetic drugs. Diabetes is a known risk factor of AD, and brain insulin signaling is deregulated in AD [74, 75]. Our preclinical studies using AD mouse models indicate that several anti-diabetic drugs, including insulin sensitizers and intranasal insulin, are promising for reduction of AD-like brain pathologies and cognitive impairment [76, 77]. Studies on the repurposing of anti-diabetic drugs for the treatment of AD was reviewed recently in detail [78]. A phase II clinical trial of intranasal insulin administration in amnestic mild cognitive impairment (MCI) and mild to moderate AD showed improved delayed memory and preserved caregiver-rated functional ability and general cognition [79]. Long-acting intranasal insulin detemir also improves cognition for adults with MCI or early-stage AD [80]. A recent randomized, double-blind, placebo-controlled phase II trial also found that the treatment of MCI or mild to moderate AD patients with daily intranasal regular insulin for two to four months improved memory associated with preserved brain volume on MRI and reduction in the tau-P181/$A\beta_{42}$ ratio [81]. Three GLP-1 (glucagon-like peptide 1) analogs, which are used for treating diabetes, have shown *in vivo* benefits in mouse AD models [82] and potential therapeutic value in AD [83]. Liraglutide is a GLP-1 receptor agonist that can cross the blood-brain barrier [84], ameliorate AD-associated brain pathologies and improve learning and memory in animal models [85–87]. This anti-diabetic drug prevented the decline of brain glucose metabolism, synaptic dysfunction, and disease evolution of AD in a 6-month small clinical trial [88]. A multicenter randomized double-blind placebo-controlled phase IIb clinical trial for AD is currently undergoing (https://www.clinicaltrials.gov/ct/show/NCT01469351). The GLP-1 analog Exendin-4 was also evaluated in a Phase II clinical trial (see https://www.clinicaltrials.gov/ct/show/NCT01255163), but the results has not been published at the time of preparation of this article.

A few clinical trials have started to evaluate drug candidates in stratified AD patients with benefits. For example, a small clinical trial testing intranasal insulin in MCI and AD patients found that the treatment facilitated recall on two measures of verbal memory in memory-impaired ApoE4 carriers [89]. Insulin also differentially modulated plasma Aβ according to ApoE genotype. Another trial testing the long-acting intranasal insulin, detemir, in MCI and AD cases found that the treatment enhanced memory for ApoE4 carriers but worsened it for non-carriers [80]. In a recent prevention trial for MCI and dementia, the subjects were stratified into four cohorts on the basis of age, ApoE genotype, sex, education, family history of dementia, vascular risk, subjective memory concerns, and baseline cognitive performance [90].

CONCLUSIONS AND PERSPECTIVES

To date, most pharmacological research is driven to discover highly selective drugs. This strategy has failed to develop any drugs that can slow down or stop the progression of AD. The recognition of the multifactorial nature of AD warrants a paradigm shift of AD drug development from a single target into multiple targets, either with the MTDL approach or the cocktail approach. The therapeutic potential of multi-targets for the treatment of complex neurodegenerative diseases like AD must be recognized. Patient stratification and the use of precision medicine model will certainly benefit both single and multi-targets AD drug discovery. While there are many potential targets for disease-modifying drugs, it is important to prioritize and test which combinations will work. It seems logical that the pathways involved in synaptic and neuronal loss, rather than the deficiencies caused by cell death or AD lesions, must be targeted in order to slow down or reverse the disease progression. Of course, targeting a combination of both would theoretically relieve symptoms and prevent further neuronal loss. Therefore, combination of pharmacophores interacting with both symptomatic and disease-modifying targets is highly justified for the initial research of MTDLs for AD.

Co-administration of several drugs is an alternative approach to treat multifactorial diseases like AD. It could be a more useful therapeutic option than designed multiple ligands. This approach might even have to be employed for AD drug clinical trials, since all single AD drug clinical trials have failed to date.

The completion of human genome study and recent advances of brain imaging and biomarkers have made the stratification of AD patients for both clinical trials and future treatments not only possible but also practical. Computational and mathematical models based on individual genomic, epigenomic, neuroimaging, and biomarker data can optimize the stratification of AD patients for better therapeutic outcomes. These models can also serve for the precision medicine model for individual AD patients for customized medical treatment.

An international Alzheimer's Precision Medicine Initiative (APMI) was recently established through a collaboration of leading interdisciplinary clinicians and scientists devoted to the implementation of precision medicine model for fighting against AD [91, 92]. The successful implementation of this model in AD will likely result in breakthrough therapies with optimized safety profiles, better responder rates and treatment responses.

Development of an effective drug for treating AD is clearly very challenging. Our experience indicates that there is no simple way of searching for AD therapy. The recognition of the multifactorial hypothesis of AD and the consideration of using the multi-targets approach gives hope for developing new and effective therapy for AD.

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Dementia Research: Populations, Progress, Problems, and Predictions

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Abstract. Alzheimer's disease (AD) is a clinicopathologically defined syndrome leading to cognitive impairment. Following the recent failures of amyloid-based randomized controlled trials to change the course of AD, there are growing calls for a re-evaluation of basic AD research. Epidemiology offers one approach to integrating the available evidence. Here we examine relationships between evidence from population-based, clinicopathological studies of brain aging and a range of hypotheses from all areas of AD research. We identify various problems, including a lack of systematic approach to measurement of clinical and neuropathological factors associated with dementia in experimental and clinical settings, poor understanding of the strengths and weaknesses of different observational and experimental designs, a lack of clarity in relation to disease definitions from the clinical, neuropathological, and molecular perspectives, inadequate characterization of brain aging in the human population, difficulties in translation between laboratory-based and population-based evidence bases, and a lack of communication between different sections of the dementia research community. Population studies highlight complexity and predict that therapeutic approaches based on single disease features will not be successful. Better characterization of brain aging in the human population is urgently required to select biomarkers and therapeutic targets that are meaningful to human disease. The generation of detailed and reliable evidence must be addressed before progress toward therapeutic interventions can be made.

Keywords: Age, Alzheimer's disease, amyloid-β protein, amyloid-β protein precursor, experimental design, population study, risk factors

INTRODUCTION

Alzheimer's disease (AD) is a complex, clinicopathologically defined dementia syndrome leading to cognitive impairment [1] and is thought to be the most common form of dementia in the older population. There is no accepted cause and the search for therapeutic interventions continues without much success [2]. Many clinical features of AD are shared with other dementing disorders and a clinical diagnosis of AD is uncertain [1, 3]. Clinical diagnosis is confirmed after death neuropathologically by the deposition of amyloid-β protein (Aβ) as plaques and cerebral amyloid angiopathy and the microtubule associated protein tau as neuritic plaques, neurites, and neurofibrillary tangles [4–6]. Familial AD (FAD), accounting for 1% of cases in populations [7], is defined by possession of a fully penetrant mutation in either the presenilins (PS) (*PSEN1* and *PSEN2*) or the amyloid precursor protein (*APP*) [8], whereas sporadic AD (SAD), associated with a range of factors including increasing age [9], possession of the *APOE* ε4 allele [10], vascular disease [11, 12], and metabolic syndromes [13], has no qualitative diagnostic feature.

Following the recent failures of amyloid-based randomized controlled trials to change the course of AD, there are growing calls for a re-evaluation of basic AD research. Epidemiology offers one approach to integrating the available evidence to guide dementia

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research strategy. Here we critically review relationships between evidence from population-based, clinicopathological studies of brain aging and a range of hypotheses from all areas of AD research to examine how the various hypotheses relate to evidence and how they may be combined to rationally select avenues of future investigation. We highlight significant areas of uncertainty in dementia research and suggest that population studies are essential to place evidence generated to date from different study designs including laboratory based, clinical trials and population studies, in a meaningful context.

AN EPIDEMIOLOGICAL, POPULATION-BASED APPROACH TO AD RESEARCH

Epidemiological approaches to AD research (Fig. 1) aim to make reliable generalizations about A) the understanding of disease processes and B) the efficacy of AD interventions [14]. We will examine these two areas separately. Observational population-based studies of aging with a brain donation program [15] are required for the characterization of AD in humans and are vital to hypothesis testing, biomarker validation, and identification of trends over time [16]. Population studies may include the whole defined population of a given geographical area in a given time period [17] or include a subset (a defined cohort) of participants that is representative of the entire population ('population-based cohort studies') [18]. These studies require a clear population provenance, with known sampling including consideration of non-participants, longitudinal attrition, and clearly described acquisition of data during life and after death. Such studies' generalizability can only be known with such information. In addition, results need to take into account age, gender, ethnicity, and other key sociodemographic features which might influence their interpretation. There are only six such studies worldwide [15] (Table 1), including Cambridge City over 75 s Cohort (CC75C) [19, 20], the Cache County study [21, 22], the Cognitive Function and Ageing Study I (CFAS) [23, 24], the Honolulu Asia Aging study (HAAS) [25, 26], the Hisayama Study [27–30], and Vantaa 85+ [31]. While each population study is unique and depends on the population from which it is drawn, results arising from these studies are generalizable at the population level and where population studies agree, these results can be understood as reliable.

Population studies are an essential resource to understand how emerging hypotheses relate to human expression of disease and to rigorously assess biomarkers and therapeutic interventions. However, they are expensive and of the 6 studies included here, only two have continuing core funding. These are in Hawaii (USA) and Hisayama (Japan), with only the Hisayama study including both genders. The remaining four no longer have any core funding and have therefore lost their previous ability to respond efficiently to new research opportunities despite the decades spent in creating them. Many excellent population-based studies exist, e.g., [33], but only those noted above have brain donation associated with them.

Population studies aim to minimize selection bias; however, the lack of selection with reference to AD due to a lack of qualitative diagnostic features in the older population, can lead to lower than expected estimates of relationships due to information bias including problems with inaccurate measurement, missing data, and poor or changing diagnostic criteria [14, 34]. The impact of factors such as attrition, non-participation, and survivor bias must also be carefully assessed [35]. Age, the biggest risk factor in SAD, has been shown to alter the associations between pathological features and dementia status [9] and therefore findings from younger cohorts may not translate to older cohorts for all disease associations. Population studies are challenging in terms of participant recruitment, longitudinal follow up with stable protocols over decades, and the organization of tissue donation and storage. The engagement and contributions of the participants themselves is invaluable to the success of these studies.

A range of other study designs contribute to dementia research in humans. Cases and controls are often selected on the basis of presence of amyloid with MRI, with PiB positron emission tomography (PET), presence of biomarkers of tau or Aβ and after death, the neuropathological Aβ or tau deposition, leading potentially to quasi-circular experimental designs where the associations between disease and neuropathology are stronger than should be expected due to selection bias. Another common study design, autopsy series, may be biased toward younger age groups and rely on attendance at specialized clinics with brain donation infrastructure whereas older people with dementia may be cared for in the family home or a care home without regular or indeed any attendance at a memory clinic for diagnosis and care with implications for interpreting any results

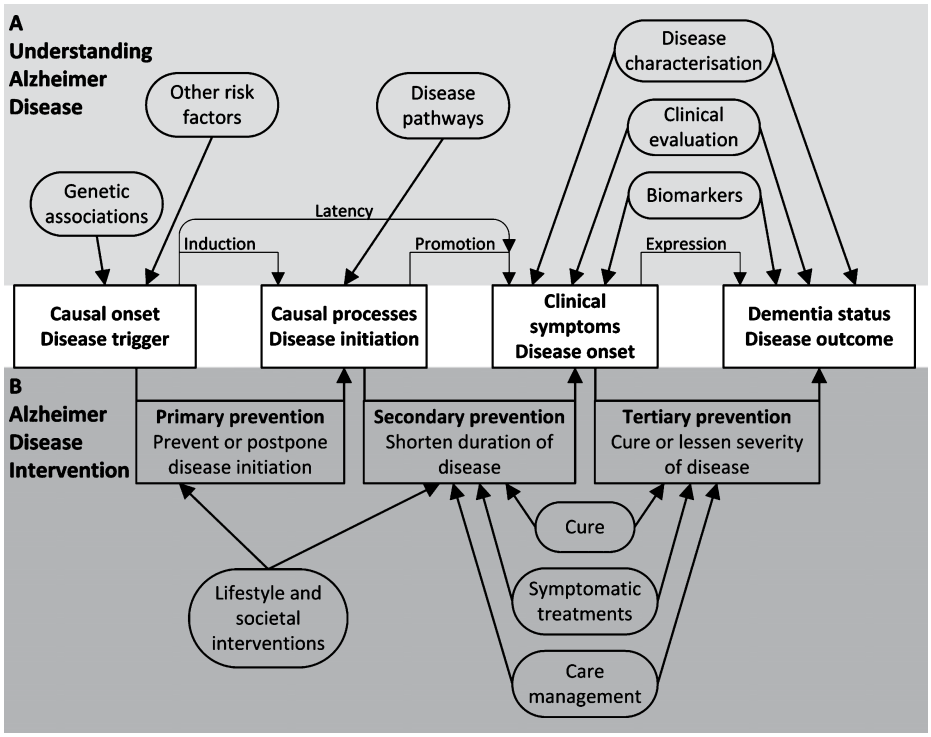

Fig. 1. Epidemiological approach applied to AD (adapted from [14]). A) Understanding AD: genetic and other risk factors trigger disease (induction) and initiate causal disease pathways leading to disease onset (promotion) with an unknown latency in AD that could be decades. Clinical dementia status is the disease outcome (expression) in AD. Clinical evaluations including cognitive function and associated genetic factors, molecular biomarker and neuropathological distributions contribute to disease characterization. B) AD interventions: primary, secondary and tertiary interventions that prevent, postpone, shorten, ameliorate or cure build on the understanding of AD pathways and can involve preventative lifestyle interventions, symptomatic treatments, management of care and ultimately aim to find a cure.

Table 1
Core funding status of population studies of aging with a brain donation program [15]

Study	Year	Selection criteria	Core Funding
CC75C (Cambridge, UK)	1985	Over 75s living registered with five geographically and socially representative GP practices in Cambridge UK - 92% response rate. Baseline sample 2166 individuals [19, 20]	No
Cache County (Utah, USA)	1995	Over 65s living in Cache County Utah - 90% response rate. Baseline sample 5092 individuals [21, 22]	No
MRC CFAS (multicenter, UK)	1989–1993	Over 65s from six areas in England and Wales UK centers (including Liverpool) random sampling from complete primary care population registers – 80% response rate. Baseline sample over 18000 individuals [23, 24, 32]	No
HAAS (Hawaii, USA)	1991	All men born between 1900 and 1919 living on the island of Oahu – 80% response rate. Baseline sample 8006 individuals in the HHP (community-based cohort study) established in 1965. 3734 individuals participated in HAAS study [25, 26]	Yes
Hisayama (Japan)	1985	Over 65s non-demented living in Hisayama Japan – 100% response rate. Baseline sample 828 [27–30] (During 7 years, 214 deceased cases; 176/2014 autopsies; there were 1266 autopsies between 1986 and 2014)	Yes
Vantaa 85+ (Finland)	1991	Over 85s living in Vantaa Finland – 92% response rate. Baseline sample 601 individuals [31]	No

[36]. Community-based study designs are population derived but are not population representative. One example, the 90+ study, included all those accepted in a specific retirement community; however, because the criteria for acceptance into that community are unknown, the relationships between this study and

the general population cannot be quantified [37]. All human-based experimental designs therefore have limitations. In combination, these approaches can be used iteratively to generate and then test hypotheses to clarify those areas of uncertainty that remain. All study types can contribute to the selection of valid therapeutic targets, with each study type having different biases, some more quantifiable than others, that impact on the precision of their findings and to which populations any findings may be applicable.

PART A: UNDERSTANDING ALZHEIMER'S DISEASE

The key to understanding neurodegenerative disorders such as AD is to be able to translate evidence between genetic, molecular, neuropathological, and clinical domains in a clear and comprehensive way. Such work ideally requires integration of biomarkers measured during life (functional and structural imaging, blood, CSF, other biomaterials) and then examination of the brain after death. *In vivo* biomarkers still require validation through longitudinal follow up for clinical progression (or not) and what is found in the brain at death.

Many hypotheses to account for risk factors, causal processes, disease initiation, and disease progression in AD have been proposed (Tables 2–4) and can be broadly grouped by main area of focus within genetic, molecular, cellular, and physiological levels (Fig. 2). While this simplistic approach is useful to illustrate the breadth of areas contributing to the understanding of AD, nearly all areas and levels considered here involve multiple avenues of cross-talk and feedback via common cellular signaling pathways and some factors in Table 2 may be relevant in more than one area. These signaling pathways contribute synergistically to a dynamic and iterative homeostatic system spanning the entire body that underlies normal functions including cognition. This interconnectedness alone suggests that an approach based on considerations of complexity has great value [38, 39]. While we aim to consider many diverse areas to illustrate the issues, our approach cannot be completely comprehensive and some areas will necessarily be covered only briefly.

Critical appraisal of the hypotheses in relation to the evidence from population studies

AβPP and FAD related

Several hypotheses relate to understanding the genetic and molecular evidence associated with dementia (Table 2). While the amyloid cascade hypothesis (ACH) focusing on the contributions of Aβ to AD has held a dominant position for decades, it has never been fully accepted and concerns remain [2, 280]. Alternative hypotheses focused on understanding molecular and genetic evidence include the presenilin hypothesis (PSH) [43, 44], which emphasizes loss or altered γ-secretase function and the amyloid-β protein precursor (AβPP) matrix approach (AMA) [48–52], which considers the dynamic behavior of the entire AβPP proteolytic system as a synergistic whole, have not been investigated in the same degree of detail as the ACH.

Understanding genetic evidence from FAD is vital as mutation in *APP* or *PSEN1* and *PSEN2* is a qualitative diagnostic feature. Rather than highlighting a fundamental disease process involving Aβ as suggested by the ACH, the genetic evidence can also be interpreted from the perspective of the AMA as highlighting various interactions and pathways, supporting the idea of AD as a syndrome of different but related pathways, not all of which may be relevant to SAD. Differences in levels of $A\beta_{40}$ and $A\beta_{42}$ [281] and differences in the AβPP beta carboxy terminal fragment [46] between *PSEN* associated FAD and SAD support this multiple pathways approach. These differences make a definition of AD at the molecular level difficult.

The mutations could be investigated from a population perspective, where possession of a particular mutation defines a distinct population which is rigorously characterized in terms of all the proteolytic fragments arising from the AβPP proteolytic system and careful analysis of how the distributions of proteolytic fragments relate to disease features such as age at onset, specific clinical features such as seizure, etc., and neuropathological characterization. Families with these mutations represent a unique and invaluable resource to investigate this complex proteolytic system as the various mutations represent natural knock in or complete/partial knock out models that are more easily translated to human disease than laboratory-based, reductionist investigations. Detailed investigations are required to identify which disease features and pathways are shared in FAD and SAD and which are unique to specific mutations.

Evidence from population studies relating to neuropathological Aβ deposition does not illustrate a straightforward correspondence that would unquestioningly support the ACH and instead finds that the relationships between Aβ deposition specifically,

Table 2
Genetic evidence from population studies listed in Table 1

Hypotheses	Main initiating factor	Evidence from population studies listed in Table 1
ACH [40]	Aβ over-production drives disease	Complex relationships between dementia status and amyloid neuropathologies do not fully support the ACH in the population [13, 20, 24, 30, 31, 41, 42]
PSH [43–45]	Altered PS function drives disease	Not fully tested in the population - mutations may affect AβPP proteolysis differently in FAD and SAD [46]; *PSEN1* E318G may not be a significant risk factor for AD in population even when combined with *APOE* ε4 [47]
AMA [48–52]	Dysregulated flow through the AβPP proteolytic system from any cause contributes to disease	Not tested - population evidence is compatible with multiple pathways to disease suggested by the AMA
Effects of *APOE* ε4 [53–56]	Possession of *APOE* ε4 allele increases dementia risk via interactions with Aβ and via its roles in cholesterol homeostasis	*APOE* ε4 allele associates with greater dementia risk [57–63] and is associated with Aβ deposition in cognitively normal older old [59] and with AD-type pathologies [64] but not with vascular dementia [60, 64]; *APOE* ε4 associated with accelerated onset but not lifetime risk [65] and effects may reduce with age [66]; one copy of the *APOE* ε4 allele reduces the neuroprotective astroglial response to Aβ plaques [67]; *APOE* ε4 associated with elevated cholesterol [68]; may moderate the associations between hypertension and cognitive function [69]; *APOE* ε4 modifies the associations between insulin resistance and NP formation [41]; *APOE* and *APOCI* loci associated with dementia in younger but not older late-onset cases [70, 71]; *APOE* allele modifies relationship between alcohol consumption, smoking and cognitive decline [72]
Other genes and genetic interactions		Gene-gene interactions may be important AD [73]; various genes not associated with dementia, e.g., α-1 antichymotrypsin (*SERPINA3*), angiotensin-converting enzyme (*ACE*) and methylenetetrahydrofolate reductase (MTHFR) [10, 74] and two polymorphisms of β-secretase 1 *BACE1* [75]; Various genes may be risk factors for dementia, e.g., α-globin transcription factor (*TFCP2*) [76] and a common variant in Enhancer of filamentation 1 (*NEDD9)* [77]; *TREM2* R47H variant is risk factor for AD [78]

ACH, amyloid cascade hypothesis; AD, Alzheimer's disease; AMA, AβPP matrix approach; Aβ, amyloid-β; AβPP, amyloid-β protein precursor; FAD, familial Alzheimer's disease; NP, neuritic plaques; PS, presenilin; PSH, presenilin hypothesis; SAD, sporadic Alzheimer's disease.

AD-related pathology in general, and dementia are complex [13, 20, 24, 26, 28, 30, 31, 41, 42]. This evidence fundamentally questions the relevance of the ACH to the understanding of AD in the older population where most dementia occurs.

Cellular systems and functions

Hypotheses relating to the contributions at the level of cellular systems and functions (Table 3) such as Ca^{2+} regulation [79, 80], neurotransmitters [82–89], cholesterol homeostasis [53–56], mitochondrial functions [106–113], oxidative stress [117–121], immune system [126–128], senescence pathways [50, 146–149], synaptic plasticity [156–158], metal ion homeostasis [160, 161], and the cell cycle [164–166] are inter-related by multiple intra- and extracellular signaling pathways. Different cell types may have different organizations of signaling cascades and may express different arrays of receptors so that neuronal or glial subtypes, may not respond the same way to the same stimulus and further, homeostatic responses over time may depend on the integration of multiple stimuli and regulation by complex feedback pathways.

Evidence from pathological, epidemiological, and genome wide association studies implicate a wide range of cellular processes in AD. However, target identification has not been straightforward. Here we illustrate challenges faced by the AD research community using cholesterol homeostasis in AD [53–56, 105] as an example. In the population, the *APOE* ε4 allele, involved in cholesterol transport, has long been recognized as a significant risk factor for AD [57–63], is associated with elevated cholesterol [68] and with AD type pathologies [59, 64] but not with vascular dementia [60, 64].

Table 3
Evidence from population studies listed in Table 1 relating to hypotheses and features relating to cellular systems and functions

Hypotheses	Main initiating factor	Evidence from population studies listed in Table 1
Ca^{2+} regulation [79, 80]	Imbalances in calcium regulation drive disease processes	Calcium dysregulation associated with AD pathology [81]
Neurotransmitters and network connectivity [82–90]	Imbalances in neurotransmitters drive loss of network connectivity and alter cognitive function	α-synuclein associated with dopamine: Lewy body dementia is related to abnormal behavior and deposition of α-synuclein; α-synucleinopathy is common in older people and associated with AD-type pathology and dementia [91, 92] though it does not increase dementia risk [93]; aging and AD-related pathologies interact with Lewy body pathology [94, 95]; increased α- and γ-synuclein proteins in cerebrospinal fluid from aged subjects with neurodegenerative and vascular changes [96]; Lewy bodies associated with loss of t-SNARE synaptic protein complex, MAP2 and α-synuclein [97]
Cholesterol and lipid homeostasis [53–56]	Disruption of cholesterol homeostasis drives disease	Abnormal lipid metabolism is associated with AD plaque-type pathology [98]; statins may delay functional decline [99] but evidence is conflicting [100] and effect was not supported in systematic review [101]; elevated late life HDL cholesterol associated with NP and NFT [102]; different lipoprotein components of cholesterol may be differentially associated with dementia [103]; reduced serum cholesterol levels may be associated with development of dementia [104]; cholesterol pathways are etiologically involved in dementia [105]
Mitochondrial function [106–113]	Disrupted mitochondrial functions drive disease	Reduced risk of AD for individuals with mtDNA haplotypes H6A1A and H6A1B [114] and is associated with mitochondrial copy number [115]; tRNA(Gln) 4336 mitochondrial DNA variant not associated with dementia [116]
Oxidative stress [117–121]	Oxidative stress is increased in the AD brain and this initiates synaptic dysfunction and neurodegeneration	Anti-oxidant use may be protective [122–124]; white matter lesions associated with markers of oxidative DNA damage and DNA damage response in glia [125]
Immune system [126–128] and infection [129–131]	Aberrant immune signaling and neuroinflammation contribute to disease; Various pathogens including *Chlamydophyla pneumoniae*, *herpes simplex* virus type-1, human immunodeficiency virus and *spirochetes* promote AD	Inflammatory factors associate with age and neuropathology [132, 133] and ApoE allele [124]; long term NSAID use may be protective [134, 135]; inflammation precedes cognitive impairments by decades [136, 137] and immune system may be etiologically related to dementia [105]; increased GFAP associates with dementia [138, 139]; increased COX-2 in neurons of CA1 correlated with AD pathology in those with but not in those without dementia [133]; adipocyte enhancer binding protein 1 protein associated with AD pathology [132]; peripheral lymphocyte subsets are related to age rather than aging-related illnesses [140]; increased glial activation associated with white matter lesions [141] and this differs by location [142]; GFAP not independently predictive of dementia but increasing gliosis precedes development of AD-type lesions [143]; innate immunity is involved in dementia [105]; reactive astrocytes close to plaques may have neuroprotective role, modified by APOE ε4 allele [67]; microglial responses are complex and diverse and respond differently to Aβ and tau in participants with and without dementia [144]; metabolic inflammasome described by the inhibitor of nuclear factor kappa-B kinase subunit beta, insulin receptor substrate 1, c-jun N-terminal kinase, and the double-stranded RNA protein kinase have roles in AD pathophysiology [145]

Senescence [50, 146–149]	Features associated with senescence such as the DNA damage response, unfolded protein response, endoplasmic reticulum stress and apoptotic pathways initiate disease	Age is the largest risk factor for AD [9, 29, 92, 150] and modifies associations between neuropathology and dementia [9]; Markers of senescence and DNA damage in glia are related to neuropathology [151]; white matter lesions associated with markers of oxidative DNA damage and DNA damage response in glia [125]; DNA damage response associated with cognitive impairments independent from AD-type pathology [152]; TDP-43 has roles in the regulation of RNA and DNA transcription and splicing [153]; neuronal inclusions of TDP-43 associated with dementia and neuronal loss [154]; TDP-43 associated with hippocampal sclerosis in old age which may act additively with AD [155]
Synaptic plasticity [156–158]	Dysregulated synaptic plasticity underlies AD	Loss of synaptic proteins follows accumulation of amyloid and tau AD-type pathology but not amyloid alone [159]; loss of t-SNARE synaptic complex associated with Lewy body pathology [97]
Metal ion homeostasis [160–162]	Disrupted metal ion homeostasis contributes to disease pathways	Higher dietary intake of some but not all metal ions reduce dementia risk [163]
Cell cycle [164–166]	Aberrant and abortive re-entry into cell cycle proliferation pathways in non-proliferating neurons contributes to disease	Not tested in population studies listed.
Cytoskeletal dysfunction	Cytoskeletal dysfunction is involved in dementia as illustrated by contributions from MAP tau [159, 167, 168]	Complex relationships between dementia status and AD associated neuropathologies with aggregated tau [13, 20, 24, 30, 31, 41, 42]; Tau pathology is associated with but does not define dementia [20, 169]; overlap of NFT densities in CA1 between non-demented elderly, AD and dementia with NFT and without amyloid deposition [170]; neuritic plaque pathology of AD associated with metabolic disorders including insulin resistance and abnormal lipid metabolism and this changes over time [30]; Braak stage hierarchy is approximate [169]; abnormality in cytoskeletal function marked by tau [159]; hippocampal tau pathology is related to neuroanatomical connections [171]; astrocyte 4R tauopathy tau pathology is common in the aging mesial temporal lobe, is independent of AD-type pathology, does not correlate with dementia. and may be age-related 4R tauopathy that includes oligodendrocytes and argyrophilic grains [172]; tau is associated with granulovacuolar degeneration [173]; neuropil threads develop hierarchically in parallel with neurofibrillary tangles and are as predictive of dementia as NFT Braak staging [174]

AD, Alzheimer's disease; GFAP, glial fibrillary acidic protein; NFT, neurofibrillary tangles; NP, neuritic plaques, NSAID, nonsteroidal anti-inflammatory drugs.

Table 4
Evidence from population studies listed in Table 1 relating to hypotheses and features relating to physiological systems and behavior

Hypotheses	Main initiating factor	Evidence from population studies listed in Table 1
Gender	Dementia may be different in men compared to women	Prevalence of cognitive and functional impairment is higher in women [150, 175]; response to dementia medication may be different in women compared to men [176]; functional disability associated with stroke in men and AD in women [177]; Health Related Quality of Life associates differently in men and women [178];
Vascular system [179–184]	Vascular abnormalities such as hypertension, small vessel disease and breakdown of the blood-brain barrier precede neurodegenerative features and drive other disease associated processes	Hypertension contributes to dementia risk [11, 185–189] but not when treated [69, 188, 190, 191] though this may be VaD not AD-related [192]; midlife hypertension is associated with AD-type pathology [189], white matter lesions and atrophy [193], compromised vascular integrity, CAA and impaired Aβ clearance [194]; vascular pathology associated with neuropsychiatric symptoms [195]; midlife systolic blood pressure predicts reduced cognitive function in later life [192, 196]; other vascular factors including atrial fibrillation, angina, small vessel disease, white matter lesions, etc., associated with AD and aging [11, 12, 92, 186, 197–202] and MCI [203], though evidence is conflicting for some [29, 185, 187]; atrial fibrillation associated with stroke not dementia [204] however stroke is associated with late life cognitive impairment [205]; beta blockers may delay functional decline [99, 206]; diuretic use may be protective [186, 207]; MRI microbleeds in basal ganglia may be associated with ischemic small vessel disease rather than hemorrhage [208]; cardiovascular factors associated with vascular dementia [209] and microinfarcts [12]; microinfarcts in cortex are associated with dementia [210–212]; subcortical microinfarcts associate with mobility [212]; hypoperfusion associated with white matter lesions [213]; blood-brain barrier dysfunction associated with white matter lesions [141, 214];
Traumatic Brain Injury	TBI events contribute to increased risk of dementia	TBI may predict progression in AD [215]
Delirium		Delirium is a risk factor for dementia [216, 217]; risk of delirium increases with severity of pre-existing cognitive impairment and neuropathology [218]; additional pathologic processes specifically relate to delirium [219]
Psychological stress	Stressful events contribute to increased risk of dementia	Child death in early adulthood associates with cognitive decline in late life [220]
Diabetes [221–223]	Metabolic syndromes contribute to dementia, VaD and AD	Diabetes contributes to dementia risk [11, 41, 185, 202, 224–227]; abnormal astrocytic insulin pathways in AD [228]; both low and high levels of insulin are associated with increased risk of dementia [229]; type 2 diabetes associated with increased risk for vascular and AD-type pathologies [230]; insulin resistance associated with NP formation and modified by *APOE* ε4 [41]; expression profiles of diabetes-related genes in AD brains related to AD pathology independent of peripheral diabetes-related abnormalities [224]; duration of diabetes is risk factor for brain atrophy particularly in hippocampus [231]
Social engagement	Social engagement supports cognitive functions via a variety of pathways	Low social engagement associated with risk of dementia however, levels of late-life social engagement may already have been modified by the dementing process [232]; social vulnerability associated with cognitive decline [233] but associations are complex [234]
Exercise	May relate to various homeostatic systems and vascular health	Increasing physical activity may be protective or delay onset of dementia [235–237] however, effects may be mild and current evidence is inconclusive [238]
Diet	May relate to various homeostatic systems	Higher milk and dairy intake reduced the risk of dementia in Japanese [239]; Vitamin E and C supplements in combination associated with reduced prevalence and incidence of AD [122, 240]; B vitamins not related [241]; diet quality is associated with better cognitive test performance [242]; whole grains, nuts and legumes may be neuroprotective [243, 244]; moderate midlife alcohol associated with better cognitive function in later life [245]; however some results are conflicting [246]; higher caffeine intake associated with lower odds of AD-type, microvascular ischemic lesions, cortical Lewy bodies, hippocampal sclerosis and generalized atrophy [247]; higher dietary intakes of potassium, calcium, and magnesium reduce risk of all-cause dementia [163]; weight loss is associated with dementia and begins before onset of clinical syndrome [248]

Continued

Table 4
Continued

Hypotheses	Main initiating factor	Evidence from population studies listed in Table 1
Cognitive reserve [249, 250]	Education and social activity may contribute to lifelong cognitive reserve that acts to mediate the effects of pathology on clinical expression of AD	Education [11, 185, 250] is protective though evidence is conflicting [251, 252]; sex, ethnicity, and lifestyle factors may significantly influence cognitive reserve [155]; various features of active cognitive lifestyles in combination are protective in relation to dementia incidence [253]; maintenance of cognitive health may be supported by a healthy and active lifestyle, in later life [237] however, these association may not persist into very old age [254]; cognitive reserve may moderate associations between mood and cognitive function [255]
Co-morbidity	May relate to aging and senescence	Comorbidity may have roles in progression of cognitive impairment and dementia [256–258]; Mid-life renal function associated with dementia and cognitive decline [259]; frailty is associated with cognitive decline [260–262]; functional disability associates with stroke in men and dementia in women [177]; tooth loss is associated with dementia [263]; hypothyroidism is associated with cognitive impairment as are high levels of free thyroxin without thyroid disease [264]; poor mobility associated with lower cognitive performance and increased cognitive decline [251]; good general health associated with lower risk of cognitive impairment [265–268]
Metabolic reserve [269–271]	Adaptive responses to perturbations in cellular systems protects against declining cognition; reserves may be associated with behaviors such as good diet and exercise or genetic such as super-agers	Exercise [28, 185], and good diet [242, 243, 272] are protective; smoking contributes to AD risk [273–275]; B vitamins may be unrelated to cognitive decline [241]; elevated plasma homocysteine levels associated with cerebrovascular and neurofibrillary pathology [276]; early [277, 278] but not late [279] hormone replacement may be protective in women

AD, Alzheimer's disease; Aβ, amyloid-β; CAA, cerebral amyloid angiopathy; MCI, mild cognitive impairment; NP, neuritic plaques; TBI, traumatic brain injury; VaD, vascular dementia.

Abnormal lipid metabolism as defined by high levels of total cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol was associated with neuritic plaques but not neurofibrillary tangles [98], elevated late life HDL cholesterol was associated with both neuritic plaques and neurofibrillary tangles [102] and development of dementia may be marked by reduced serum cholesterol levels [104] supporting an etiological role for cholesterol homeostasis in AD. However, translating this approach into meaningful interventions to prevent, delay or control disease progression has not yet been successful. Statins were found to delay functional decline in one population study [99] but not in another [100] and the effect was not supported in systematic review [101]. The complexity and interconnectedness of the immune and cholesterol systems as illustrated by the multiple roles of ApoE in both [105, 282] and the additional roles of cholesterol in the cardiovascular system with implications for the development of dementia, listed in Table 4, suggest that we need to clarify our understanding of the relationships between these systems and AD progression before therapeutic targets aimed at cholesterol homeostasis can be identified with any certainty.

Physiological systems and behavior

As with hypotheses relating to cellular systems and functions, those relating to whole physiological systems and behavior listed in Table 4, such as the vascular system [179–184], diabetes, infection [129, 130], stressful life events [283], cognitive [249, 284] and metabolic [269–271] reserve are also connected by multiple pathways and additionally may be affected by human lifelong experience [285], wider genetic background and environmental factors [286, 287]. Population studies show that general health relates to cognition [265–268], comorbidity is more serious in those with dementia [256], sociological/economic factors are important [267, 288, 289], that dementia incidence and prevalence estimates change over time [30, 42, 290] and differ between populations [29, 291] and by sex [155, 292, 293].

Further, the prevalence of cognitive and functional impairment may be more common in women [150, 175] and some studies report cognitive decline is faster in women than men [293]. Functional decline is associated more with stroke in men and AD in women [177] and responses to dementia medication may be different in women compared to men [176]. This suggests that gender differences may represent differ-

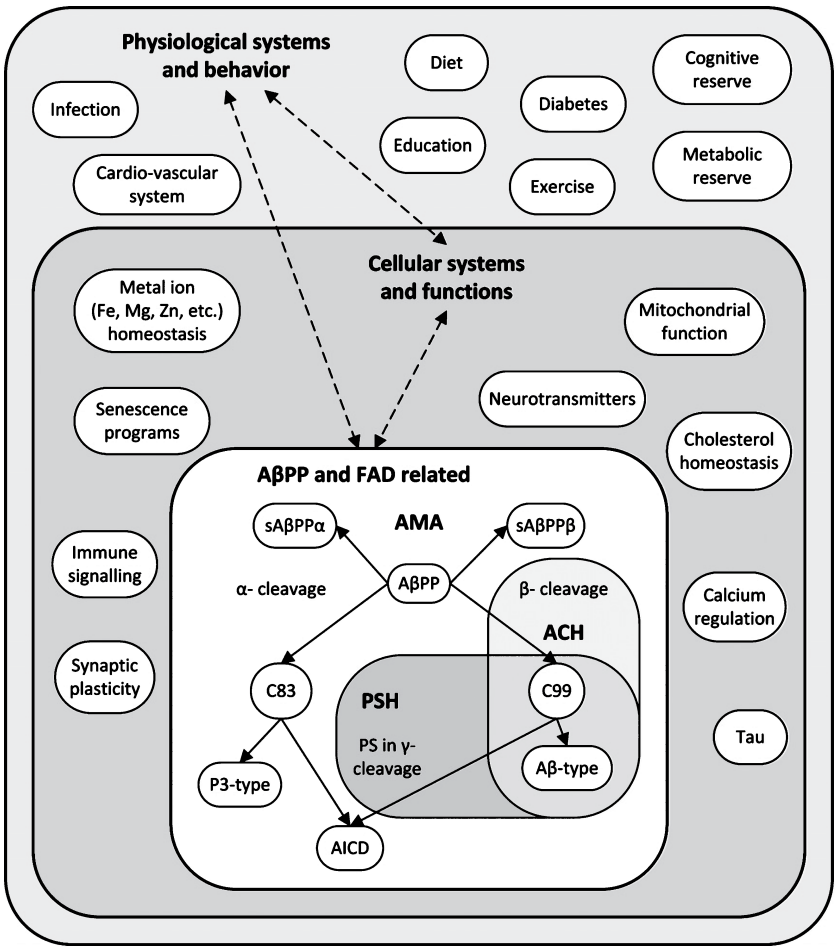

Fig. 2. AD hypotheses grouped by main areas of focus. Yellow: ACH - main area of focus is β-cleavage and main outcomes investigated are levels of Aβ; orange: PSH – main area of focus is γ-cleavage and main outcomes investigated are levels of Aβ overlap with ACH indicated by area of light orange; white: AMA - main area of focus is whole AβPP cleavage system main outcomes would include all proteolytic fragments including Aβ; light blue: various cellular system and functions – main outcomes depend on system being investigated and include levels of Aβ; dark blue: Various physiological and behavioral systems - main outcomes depend on system being investigated and include levels of Aβ.

ent disease pathways in men and women that further complicate the search for therapeutic intervention and the design of randomized controlled trials.

All the above taken together suggests that AD is a complex disorder relating to the whole person and the context in which we live and that focus on one particular part requires an understanding of the wider context.

Integrating the available evidence

In order to fully understand progress so far and future directions in dementia research, it is essential that evidence is translatable between clinical studies in humans, neuropathological diagnostics, and molecular investigations. This evidence must be reliable and assumptions must be transparent and testable if we are to be able to tease apart the complexities.

We have previously suggested that the AMA [48–52] (Fig. 3) provides a flexible framework with which different areas of dementia research can be inter-related and understood. Rather than focusing on one small part of the AβPP proteolytic system as seen in the ACH with Aβ, the AMA focuses on the complexity of the AβPP proteolytic system as a dynamic whole and emphasizes the contributions from wider cellular systems that affect the balance between the AβPP cleavage pathways via regulation of α-, β-, γ-, and other cleavages. Further, the AMA suggests that the AβPP proteolytic system feeds back

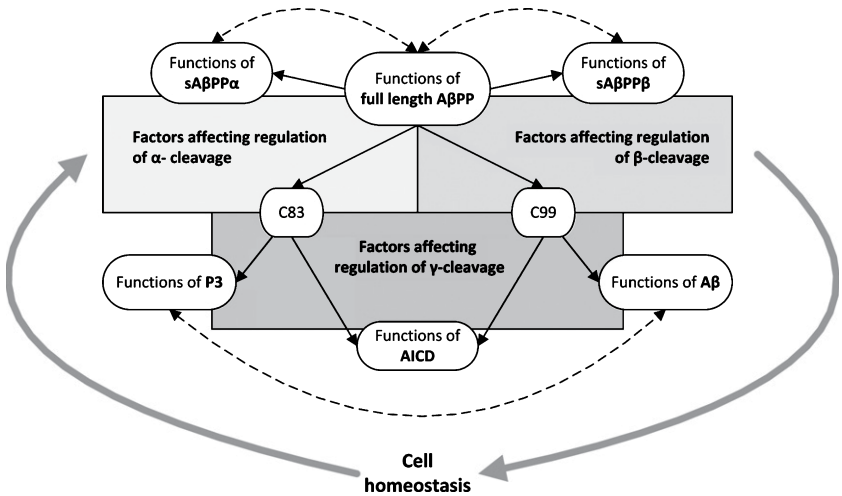

Fig. 3. The AβPP matrix approach. Solid black lines represent cleavage pathways; dotted lines represent synergistic interactions of full length AβPP, sAβPPα, and sAβPPβ (top) and P3 and Aβ (bottom). Solid grey lines represent complex synergistic homeostatic interactions between wider cellular systems and the AβPP proteolytic system. Other cleavages, e.g., BACE2 leading to sAβPPβ' and Aβ', general catabolism of all fragments and caspase cleavage not shown.

to these wider cellular systems via the ratios of all the proteolytic fragments leading to a dynamic cyclic system capable of coordinating cellular responses. The fragments interact synergistically with wider cellular systems in agonistic and antagonistic manners both intra and extracellularly. This approach to understating the AβPP proteolytic system is compatible with recent work showing that cellular communication relies on combinations of small binding proteins, such as Aβ- and P3-type peptides, and receptor expression rather than absolute levels of these proteins [294]. In this respect, the AMA has the potential to integrate current evidence relating to various homeostatic systems such as cholesterol, Ca^{2+}, immune signaling, cell cycle, senescence, oxidative stress, etc., and the roles of AβPP proteolytic fragments in a way that better represents cellular functions. The AMA suggests that FAD associated mutations affect this homeostatic balance in ways particular to each mutation.

In contrast to the ACH, the AMA suggests that while Aβ has a role in disease, it is only one small part and its expression can either drive disease or be driven by other disease related factors depending on the exact disease context. The AMA is compatible with the PSH if the PSH widened consideration to all fragments released from γ-cleavage and evidence exists to suggest that the production of P3 is affected similarly to Aβ by *PSEN1* mutation [295]. The AMA is also compatible with other detailed hypotheses listed in Table 2 as it allows various factors to impact directly on AβPP cleavage, meaning that perturbations in wider cellular systems can both drive and be driven by disease pathways. As such, the AMA is a framework that allows each detailed hypothesis to be placed in relation to others in a synergistic way to see where factors converge or conflict and so allows a more flexible experimental approach to identify therapeutic targets.

PART B: ALZHEIMER'S DISEASE INTERVENTIONS

The hypotheses listed in Tables 2–4 form a network from which therapeutic targets can be identified and interventions can be designed that prevent, postpone, shorten, cure, or reduce the severity of impairment in AD (Fig. 1). It is not currently possible to tie the various hypotheses listed to pathological processes with the detail required for therapeutic intervention with certainty. The data are confounded [296] and for some hypotheses, data from population representative cohorts are inconclusive or missing. The rational assessment of which features of disease merit more detailed investigation is difficult with the current limited evidence and there are many problems that undermine current understanding in dementia research.

Problems with current dementia research strategy

Within the older population, it is not clear whether we are dealing with one generally applicable or multi-

ple sub-groups of pathways to disease. If we consider the evidence of cellular and physiological systems in the population, we understand that not everyone with dementia will share specific features, e.g., specific neuropathologies or in life factors such as possession of ApoE ε4 alleles, gender, diabetes, or hypertension, but that each factor has the potential to exert a partial pressure to modify disease expression. We are not certain that those with diabetes or contributions from other factors such as vascular disease [297] or aggregation of the TAR-DNA binding protein 43 (TDP-43) [154] will necessarily share the same therapeutic targets with those possessing an AD-associated mutation. In addition to differences in the contributions of factors such as inflammation and diabetes, gender differences suggest that at the population level, AD is poorly defined.

Progress in dementia research requires that findings are replicable within and translatable between different experimental systems. However, this presents problems for such a complex disorder. With no qualitative diagnostic feature, the diagnosis of SAD in those with dementia depends on cut off points along continua of features, such as neuropathological variables, biomarkers [298, 299], and ^{11}C-PIB PET [300] that have yet to be validated and do not always agree [301, 302]. There is significant overlap in these features between those with and without dementia [20, 170] so that the selection of cases and controls in SAD for randomized controlled trials or hypothesis testing is uncertain. Within populations, there are individuals with inappropriately high or low burdens of pathology in relation to their clinical dementia status; in CFAS, 25% of respondents were neuropathologically misdiagnosed when assessed blind to clinical status [24, 199]. This evidence suggests that while they are associated with dementia, neuropathological features alone do not clearly define AD.

The continua of diagnostic features raise concerns relating to the classification and definition of dementia and AD in the older population. Where do we place cut-off points to capture dementia diagnoses as accurately as possible and will these be the same for each study? We cannot know whether those who died with high burdens of pathology but no dementia, defined as prodromal AD, would have developed dementia if they had lived longer. Those diagnosed clinically with AD-type dementia but with no or insufficient pathology for a neuropathological diagnosis of dementia type, suggest that further pathway(s) relating to dementia remain to be found that could potentially contribute to the lack of correspondence between clinical dementia and neuropathological diagnosis in the population. Is the definition of AD in the context of laboratory-based mechanistic studies using animals or cell culture, often operationally reduced to levels of Aβ, applicable to human disease? Is there a reliable molecular definition of AD that is transferable between different experimental approaches or even the different disease categories, FAD and SAD, in humans? To what extent does poor definition of AD in the various experimental contexts contribute to lack of progress—are we investigating the same AD in all approaches? These issues relating to defining AD are fundamental to dementia research and have been raised before [303] but are as yet unanswered.

Dementia in the older old is often mixed with contributions from a range of pathologies contributing to dementia [20, 92, 304] and MCI [203, 297] and the correspondence between clinical diagnosis and neuropathological diagnosis blind to clinical status is not strong [20]. In CC75C, 85% of those with and 76% of those without dementia had sufficient AD-type neuropathology for a diagnosis of AD when assessed blind to clinical status. Multiple pathologies often contribute measures of the overall burden of dementia [20, 26, 92] making it difficult to assign causal roles to specific pathologies with certainty. Is the current strategy of targeting therapeutic interventions at single disease features appropriate?

Despite decades of research, Aβ-related pathologies have yet to be fully characterized in the human population, e.g., Aβ deposition as different plaque types [305], different sequence lengths, aggregation states, and solubilities, and their context within the wider AβPP proteolytic system have not been adequately investigated. We do not know whether pathology is a proxy for other as yet hidden processes or whether pathology is inherently neurotoxic or a mix of both. Similar issues may also apply to other neuropathological features related to protein aggregation and deposition, e.g., tau [20, 24, 31] and TDP-43 [154].

No study has yet measured the contributions of all the AβPP proteolytic fragments so there is a degree of confounding when assigning particular features of disease to any one proteolytic fragment [49, 52]. Since levels of AβPP are rate limiting, any proposed gain of function in one cleavage pathway necessarily leads to loss of function in another in this complex proteolytic system. These contributions will be confounding unless they are controlled for in experimental design. At the level

of basic science, confounding arising from cross reactivities of commonly used anti Aβ antibodies in human neuropathological diagnostics and research [296] requires urgent clarification. We need to know which specific peptide sequences released from γ-cleavage are present in amyloid deposits and CSF and how much of each specific aggregation state (monomers, dimers, oligomers, and fibrils) for each specific sequence length is present—detailed information which is entirely missing from the literature base. Definitions of Aβ in practice may not be the same between different research approaches and results from studies using different anti Aβ antibodies are not directly translatable.

Given the complexity of the AβPP system and the uncertainty surrounding anti-Aβ antibody cross reactivities, it is time to address this lack of understanding and accurately describe the AβPP proteolytic system as a synergistic whole in humans. However, the measurement of a dynamic and iteratively changing system leads to a paradox of absolute measurement at one time (cross-section) versus measurement of flow through a pathway (longitudinal). Can a measurement at one point in time, as represented by MRI, sampling biological fluids for biomarkers or examining the brain after death, adequately describe a dynamic system changing in response to multiple perturbations over various time scales, e.g., diurnal variation [306, 307]? Understanding what biomarkers or neuropathological assessments actually represent remains to be fully addressed in AD. We should not be assuming that they are directly neurotoxic and represent therapeutic targets without understanding the complex human context in which they exist. We do not yet have the techniques to non-invasively generate the evidence required to understand dementia pathways in humans. How should we reduce the complexity of human cognitive function to generate laboratory-based models that can be used to dissect the complex processes associated with cognition and its failure and how do we test whether any such reductions are applicable?

The lack of full characterization of dementia in human populations impacts on laboratory-based, between-species comparisons, e.g., given that the role and the organization of G-protein coupled receptor signaling in mice and humans differs in pancreatic islets cells [308] and up to 90% of GPRs may be expressed in the brain [309], we can ask whether G-proteins in the brain have equivalent roles and organization between species. Other differences are suggested such as the role of PS1 in human oligodendrocytes and myelination that is absent in the mouse [310]. Between species differences lead to difficulties and potential failures when directly applying results from animal research to humans. Better characterization of both animal models and human populations will lead to better experimental models, more refined therapeutic target identification, and enable a more detailed understanding of how animal research can be best translated to humans.

Standardization of methodological issues relating to tools, experimental design, scoring and measurement protocols, and reporting of results is essential in order to rationally interpret findings from various experimental approaches in a wider context. While generalizable and qualitative trends can be identified from population studies, different methods to assess dementia status, different neuropathological protocols including the use of different antibodies, different diagnostic cut-offs and different methods of analysis make detailed comparison between studies difficult.

FUTURE DIRECTIONS

We suggest that the current evidence base is too narrow and it is not possible to identify therapeutic targets that have good chances of success to change the course of disease in humans. Several issues undermine a clear research strategy in the immediate future and require clarification.

1. Agreed definitions of AD and Aβ that are transferrable between clinical, neuropathological, and molecular evidence bases are urgently required.
2. There are currently few fully accepted, standardized measures and reporting formats that allow direct comparisons between studies. While qualitative comparisons are valuable, standardization to allow quantitative comparisons, especially for molecular factors, such as specific Aβ-type peptides, is required.
3. We do not have the detailed evidence required to directly translate molecular findings between laboratory-based mechanistic studies and disease in the human population. Several stages of translation need to be developed before this can happen reliably. We need to better characterize the relationships between clinical dementia, biomarkers of AD, neuropathological diagnosis, and specific molecular features in the human

population and explore how these relate to laboratory-based experimental models.

4. Better characterization of dementia related factors in the population will increase understanding of how many AD related disease pathways are possible and which pathways share therapeutic targets. There may be groups of disease pathways that can be better defined with better characterization of human disease.
5. Hypotheses guide both experimental design and interpretation of results. The ACH effectively reduces the output of the AβPP proteolytic system to measures of Aβ. This limits considerations of complexity. We should address complexity by characterizing the AβPP proteolytic system in a systematic manner and interpret results within more flexible frameworks that reflect the complex cellular milieu.

Future dementia research strategy depends on clarifying our understanding of current evidence and identifying sources of uncertainty to be corrected. Without such detailed assessments, identifying therapeutic targets and drug discovery strategies may not have the rational basis required.

SUMMARY

AD research has been dominated by the ACH for decades with little advance in our understanding of the role of the AβPP proteolytic system as a whole in disease initiation and progression due to confounding by molecular complexity, misunderstanding of antibody reactivities, and biased experimental designs. The neglect of the PSH, relating to the contributions from γ-secretase, and the AMA, relating to the dynamic balance between all cleavage pathways and products of the AβPP proteolytic system, can be understood as a significant hypothesis bias. The genetic and neuropathological evidence emphasizes the importance of this proteolytic system in AD, and it is now time to re-assess the evidence so far to clarify our understanding.

Population studies are an unrivaled resource to better characterize the myriad factors associated with dementia and be able to translate these findings to better diagnostic protocols that are urgently needed. They also highlight complexity and predict that single therapeutic approaches based on isolated disease features will not be successful.

The importance of population studies for disease characterization, hypothesis testing, and disease marker validation has not been fully acknowledged by the wider AD research community and their essential contributions to the development of efficient research strategies are neglected. Better characterizations of brain aging in the human population will lead to a more rational selection of AD therapeutic targets that are meaningful to human disease. Biomarker validation in the human population will lead to a better understanding their relationship with disease and refine how they can be applied clinically. Research at the population level is significantly hindered by lack of core funding and without it, unquantified bias in experimental designs may mislead the research community. More flexible molecular models such as the AβPP matrix approach may contribute greatly to integrating and understanding evidence from very diverse fields of dementia research.

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Three Decades of Dementia Research: Insights from One Small Community of Indomitable Rotterdammers

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Abstract. The most commonly encountered opening sentence in scientific publications about dementia undoubtedly relates to the overwhelming burden of disease. Finding an effective preventive or therapeutic intervention against dementia has been considered the most important unmet need in contemporary medicine. While efforts on tackling this devastating disease have increased exponentially, it is difficult to imagine that in the 1980s and early-1990s, the disease did not feature prominently on any public health report. Yet, it was already then that epidemiologists recognized the growing societal burden of dementia and rationalized that dementia is not necessarily part of aging. Indeed, the conviction that dementia is pathologically distinct from aging led to various efforts in search of unravelling its risk factors and understanding its pre-clinical phase. Among the early pioneers, the population-based Rotterdam Study was initiated in 1990 clearly aiming on chronic diseases including dementia, and among this Alzheimer's disease, as one of its focus points. Ever since, the Rotterdam Study has been an important cornerstone in increasing our knowledge about dementia from an epidemiological perspective. Here, we summarize the main findings originating from this study, and put these into perspective with previous and current work in the field. With an expanding scope of the Rotterdam Study over the years, we discuss findings on occurrence, modifiable risk factors, imaging, and its genetic underpinnings. Importantly, we conclude with recommendations—or, perhaps better stated, a wish list—for future research which may help us reach our finish line: finding an effective preventive or therapeutic intervention against dementia.

Keywords: Alzheimer's disease, cohort studies, dementia, epidemiologic methods, epidemiology, neurodegenerative diseases

DEMENTIA FROM A POPULATION PERSPECTIVE: PRESENT AND FUTURE

The inception of the Rotterdam Study cohort in 1990 provided the first population-based numbers of dementia prevalence in the Netherlands [1], contributing at the time to early efforts in Europe and the US to reliably map the prevalence of dementia in the population [2].

Over 15,000 people from Ommoord, a suburb of the city of Rotterdam, now participate in the ongoing study. Apart from the 4-yearly visits to the research center, participants are under continuous surveillance via the medical records of their general practitioner (a 'gatekeeper' in the Dutch healthcare system). This provides important information on their wellbeing,

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even when frailty prevents repeated assessment at the study center, allowing reliable estimates of lifetime risks and life expectancy with disease. Roughly one in three individuals in the Rotterdam Study get diagnosed with dementia, stroke, or parkinsonism during their lifetime, and roughly 1 in 5 develop dementia, with an average age at diagnosis of 81 years [3]. In terms of life expectancy, this means that on average 6% of remaining life years at age 65 will be lived with dementia, increasing to over 35% at age 95. Such findings are needed to guide formation of health care policy and prioritizing research efforts.

Much of the recent thrive in dementia research, and its funding [4], is sparked by 'epidemic' projections, with global prevalence expected to triple by 2050 [5]. But how reliable are the forecasts? Following initial prevalence estimates, the long-term, methodologically consistent observations of the Rotterdam Study cohort have enabled assessment of trends in de occurrence of dementia over the past 27 years. In support of evidence from Rochester (Minnesota) in the United States (US) [6], we showed in 2012 that the age-specific incidence of dementia has in fact been declining [7], accompanied by larger brain volumes and lower burden of white matter hyperintensities. Alike the first reports about the rise and fall of myocardial infarction incidence in the middle of the 20th century, this triggered much debate, which is still largely unresolved in terms of factors underlying these trends [8, 9]. Nevertheless, various studies have now corroborated the declining incidence trends, at least in the US and Europe [8], highlighting that developments over the past century, be it in educational attainment, treatment of cardiovascular risk factors and diseases, or other public health developments like hygiene, have benefitted our resilience against dementia. Yet, these optimistic trends do not negate the projected growth in the number of people living with dementia due to the ageing population, and may even be offset by increases in the prevalence of obesity [10], type 2 diabetes [11], and hypertension [12]. This illustrates the importance of identifying modifiable risk factors, and unravelling their role years, if not decades, before the onset of clinical symptoms of dementia.

MODIFIABLE RISK FACTORS: KEY TO CURBING THE EPIDEMIC?

Designed to identify determinants of disease and disability in the elderly [1, 13], much of the research done in the Rotterdam Study over the past years has been aimed at identifying modifiable risk factors for cognitive decline and dementia. Unlike biomarkers, which often reflect early subclinical alterations due to the disease process and are discussed below, the identification of causally related risk factors has the ultimate aim of developing preventive interventions. The Rotterdam Study, along with other population-based studies, has shown that known modifiable risk factors are accountable for approximately 25–30% of all dementia cases [14, 15]. By calculating the so-called population attributable risk, which takes into consideration the overlap between risk factors in individuals, this implies that eliminating these risk factors from the population would reduce the incidence of dementia by nearly a third. Although complete elimination of a risk factor from the population is often unachievable, it illustrates the large burden of these well-known risk factors on public health. Albeit generally modest in effect size at the individual level [16], public health interventions that target these risk factors could greatly reduce the burden of disease at the population level. The exponentially increasing incidence of dementia with age, unlike any other disease, has large implications for the potential of preventive medicine. The vast majority of life years spent with dementia are lived in the final few years of one's lifespan, meaning that postponing the onset of dementia by merely a few years can reduce the lifetime risk and number of life years spent with dementia by up to 50%, as seen in the Rotterdam Study and beyond [17].

Despite the generally late-life onset, dementia is increasingly becoming 'a disease of mid-life', which reflects the general uncertainty regarding the earliest origin of the disease. Various risk factors, notably obesity [18] and hypertension [19], are particularly detrimental to late-life cognition when present in mid-life. The age at which people are eligible for the Rotterdam Study has dropped from $\geq$55 in the inception cohort to $\geq$40 years in the latest inclusion wave to reflect the importance of life course data [1]. Trajectories of cholesterol and blood pressure levels [20], but also clinical manifestations like depressive symptoms [21], aid in disentangling the time course and thereby mechanisms by which these are related to dementia onset. Moving onward by going further back in time, reliably tracking the life course of individuals from the prenatal phase and childhood [22] to adulthood and late-life is the next step in understanding of dementia, by linking developmental variation to neurodegenerative sequelae.

Given the importance of vascular disease in the onset of dementia, it is well conceivable that patients with heart disease are at increased risk of dementia. Thanks to improving acute treatments and secondary prevention, many patients with coronary heart disease or heart failure now live well into old age and are consequent susceptible to late-life diseases like dementia. Indeed, a history of heart disease relates to an increased risk of developing dementia, apparently independent of aforementioned risk factors, and even in the presence of subclinical myocardial infarction or cardiac dysfunction [23, 24]. This puts forward the possibility that long-term hemodynamic impairment and consequent hypoxia could be detrimental to brain health. We recently observed in the Rotterdam Study that low cerebral perfusion increases dementia risk, as well as cognitive decline in non-demented individuals, during on average 7 years of follow-up [25]. Although this does not rule out reduced perfusion due to metabolic changes in the very early preclinical phase of dementia, it does support further studies to assess whether improvements in perfusion, for example by physical activity, could be beneficial to brain health. In this context, cerebral autoregulatory mechanisms, including vasoreactivity and autonomic function [26, 27] could be vital to maintain sufficient oxygenation in the presence of disturbed flow. Further (circumstantial) evidence for a role of hypoxia in dementia etiology comes from associations of hemoglobin levels with cerebral perfusion, and long-term risk of dementia possibly pointing to a regulatory mechanism which involves either to maintain tissue oxygenation [28].

Other projects have focused on the role of lifestyle factors, beyond traditional cardiovascular risk factors, in the development of dementia. Educational attainment [14], depressive symptoms [14], and hearing loss [29], are examples of modifiable factors that may contribute to prevention of dementia [30]. In addition, physical activity is widely regarded as a protective factor against dementia. In part, this is supported by observations in the Rotterdam Study, showing protective associations up till 5 years of follow-up, but not thereafter [31]. This time-window raises the possibility of reverse causation, but could also be due to changes in behavior otherwise, the limitations of a single measurements, and the coarse nature of a physical activity questionnaire. In terms of diet, a healthy diet is generally considered beneficial, but lack of association between dietary adherence and cognitive decline [32], and dementia [33], which we observed in Rotterdam as well as in observational studies, suggests that there may be specific components only, such as represented in the Mediterranean diet [34], that have beneficial effects on cognition. In addition, observational studies vary widely in terms of intensity, frequency, and duration of exposure [35], highlighting the need for standardized quantification criteria for diet, as well as other of aforementioned lifestyle factors.

The challenge before us is to translate these observations on risk factors into biological mechanisms, in other words to treat risk factor associations as probes to advance etiological insight and ultimately facilitate preventive interventions. This regularly requires collaboration with basic and translational science, whereas advances in –omics initiatives (e.g., genomics and metabolomics) have created new opportunities to translate observations in the population to plausible mechanisms. Also, emerging measurement techniques, i.e., imaging, make it possible to examine community-dwelling individuals in more depth. The yield and further implications of these developments for population-based studies like the Rotterdam Study will be discussed in the next sections.

PRECLINICAL IMAGING IN DEMENTIA: WHAT HAVE WE LEARNED?

Already in the early 1990s, the Rotterdam Study acknowledged that thorough investigation of the etiology and the pathological mechanisms of dementia required in-depth visualization of the brain. The Rotterdam Study has always been a pioneer in introducing state-of-the-art imaging techniques into the population-based setting. These include retinal imaging, computed tomography imaging, magnetic resonance imaging, diffusion tensor imaging, and resting state imaging and the current section is dedicated to several of the most important recent findings from the Rotterdam Study [1, 36].

Brain imaging represents one of the cornerstones of current-day population-based dementia research [37]. As one the first population-based studies worldwide, the Rotterdam Study incorporated brain magnetic resonance imaging (MRI) into the core study protocol in 2005 [36]. Additionally, already in 1990 and then again in 1995 and 1999, subsets of individuals were invited for MRI. The excellent capacity of MRI to visualize brain structure and pathology, combined with its ability to assess

brain function using the properties of the cerebral circulation, positions MRI as an extremely valuable non-invasive imaging tool for population-based brain imaging.

A strong focus of the Rotterdam Study has always been to establish cerebral small vessel disease as an important substrate of the dementia process. Among the earliest findings, we showed that a larger burden of white matter hyperintensities, lacunes, and cerebral microbleeds are associated with a higher risk of dementia and mortality [38, 39]. Importantly, these markers of cerebral small vessel disease are also directly linked to the preclinical phase of dementia, evidenced by the associations with cognitive deterioration, mild cognitive impairment, and deterioration of and impairment in daily functioning [39–41]. Of note, these pathologies to accumulate more in the white matter than in the grey matter. A natural extension was therefore to identify even earlier markers of presumed vascular damage. This led to the introduction in 2005 of diffusion tensor imaging (DTI) into the MRI protocol. Indeed, microstructural changes as quantified using DTI, were found to be already present in the normal appearing white matter and to precede the formation of white matter hyperintensities [42]. These findings further established what was already known through small clinical and animal studies: white matter hyperintensities develop gradually and that those that are visible only represent a small portion of the underlying white matter pathology. In terms of clinical relevance, we described that general loss of the microstructural integrity of the white matter plays an important role in the etiology of cognitive impairment and dementia [43, 44]. Interestingly, we even showed that degenerative changes in white matter microstructure also mark health outcomes beyond the brain, including gait impairments and a higher risk of all-cause and cardiovascular mortality [45, 46].

In addition to assessments of general microstructural degeneration diffusion-weighted imaging also allows quantification of microstructural integrity of specific white matter tracts [47]. Using these techniques, we found differential patterns of degeneration in specific white matter tracts with aging. In particular the limbic, association, and commissural tracts, appeared to degenerate most prominently with aging and might represent more specific neurodegenerative markers than overall white matter atrophy or the amount of white matter hyperintensities [44, 47, 48].

Given our strong focus on the vascular pathways underlying dementia, the Rotterdam Study introduced various other modalities that comprehensively probe the cerebral microvasculature and hemodynamics. We discuss here cerebral perfusion, computed tomography (CT) imaging of the cerebral arteries, and retinal imaging.

With the introduction of our dedicated MRI-scanner in 2005, we also introduced measurements of total cerebral blood flow (i.e., cerebral perfusion) with use of dedicated phase contrast sequences [36]. In one of the earlier studies on cerebral perfusion a direct link between reduced perfusion and degenerative brain changes was shown [49], with complex underlying associations between cerebral blood flow and brain [50]. In terms of clinical significance, we also recently highlighted that cerebral hypoperfusion is associated with accelerated cognitive decline and an increased risk of dementia [25].

Toward the end of the millennium, conventional ultrasound of the carotids was the hallmark of large-vessel damage as it relates to dementia [51–53]. In 2002, the Rotterdam Study moved beyond ultrasound and added CT-imaging in a large subset of the population. In contrast to other population-based studies that introduced CT-imaging around the same time focusing on the coronaries and aorta, the Rotterdam Study had a broader scope and included visualization of intracranial vessels [54]. In several papers since, we showed that calcification in the extracranial and intracranial vessels contributes to cerebral atrophy, cerebral small vessel disease, cognitive decline and dementia [55, 56], further emphasizing the importance of vascular disease in the etiology of dementia.

Whereas cerebral perfusion and CT-imaging as discussed above provide a measure of large vessel damage, we used retinal imaging since the inception of the Rotterdam Study to directly quantify the small vessels. We found that retinal vascular calibers relate to cerebral atrophy, especially white matter atrophy, and with worse white matter microstructure [57, 58]. In addition, increasing evidence supports an association between retinal vascular changes and dementia, especially Alzheimer's disease [59].

GENETIC RISK FACTORS: DISENTANGLING THE COMPLEXITY OF DEMENTIA

In addition to modifiable risk factors that accumulate during life, part of the susceptibility to dementia

is already determined at conception by your genetic make-up [60]. Estimates for the heritability of dementia syndromes vary greatly, suggesting anything from a predominant genetic component to a minimal influence by genes [61]. While the level of heritability may differ depending on the methodological approach and the specific study population, it is clear that genes do play a role in dementia [62]. The first genetic risk factors for dementia were identified for familial forms by studying affected pedigrees [60]. However, sporadic forms, which are the most common, do not result from one specific mutation but rather they are the consequence of multiple risk increasing variants that may or may not lead to dementia depending on other genetic variants or additional environmental factors. Of these, *APOE* is the most well-known due to its large effect size in combination with a high frequency of the risk alleles in the general population [63–65].

Many candidate variants have been studied in relation to dementia, but these unfortunately rarely replicated. With the advent of large scale genome-wide association studies, robust associations have been identified between common variants and dementia [62]. Their effect sizes are modest and with odds ratios below 2.0, and generally much smaller [66]. Recently, rare variants with slightly larger effects have been identified through sequencing studies [67]. The participation of the Rotterdam Study in these discoveries was mainly by contributing samples to large consortia such as CHARGE and IGAP [68, 69].

However, the Rotterdam Study has been more actively leading initiatives to map genetic determinants of endophenotypes of dementia. While dementia is a heterogeneous syndrome resulting from a multitude of factors, endophenotypes are thought to represent a more distinct disease process that is closer to the underlying biology. For example, amyloid-β levels in plasma or cerebrospinal fluid are likely to be more specific markers for the amyloid cascade, while measures of the cerebral perfusion may indicate vascular pathways [25]. We have focused our endophenotype studies mainly on neuroimaging markers retrieved from MRI. In the Rotterdam Study, over 13,000 scans have been performed using the exact same MR machine and acquisition protocol, making it the single largest study with such data [36]. The first genetic studies of imaging markers were published in 2012, where we studied hippocampal and intracranial volume [70, 71]. We found that robust association signals could be identified in samples of around 10.000 individuals and that some of these relate to risk of dementia [72, 73]. After these initial publications, larger studies have been performed on these and additional neuroimaging measures [74–76]. We have performed genetic association studies of the volumes of other subcortical structures [77], and found that amygdala volume might be a good endophenotype for Alzheimer's disease [77]. An important development in these studies is the availability of biobanks such as the United Kingdom (UK) Biobank, which have provided a large resource of valuable data [78, 79]. Beyond the studies of these gross neuroimaging measures, we have also investigated whether novel high-dimensional imaging markers may be more informative for genetic studies. Two of these are the shape of subcortical structures and voxel-based grey matter morphometry [80, 81]. In both of these studies, it was found that there exists substantial regional variation in the heritability of these high-dimensional markers that would have been missed when looking only at the traditional measures that describe the brain roughly [82]. While the next step would logically be to perform genome-wide association studies of all these novel imaging measures, their sheer number poses a practical obstacle. For voxel-based morphometry, for example, there are 1.5 million voxels in the brain for which genome-wide association studies, with 10 million genetic variants, would result in trillions of association tests. This is computationally intensive but also makes for a stringent multiple testing threshold. To overcome these barriers, we have developed a new analytical framework, HASE, that is specifically designed for high-dimensional analyses [83]. The computational time for such a genome-wide brain-wide association study is greatly reduced from several years to only several hours. This is achieved by implementing smarter algorithms for data storage and retrieval, but also by using a novel form of meta-analysis termed partial derivatives meta-analysis [84]. In a proof of principle study within 4000 individuals of the Rotterdam Study, we performed genome-wide association studies of 7000 voxels in the hippocampi and found this to be indeed computationally feasible [83]. Interestingly, the top variant was located in a locus that has been previously associated with hippocampal volume in a much larger sample. Larger genetic studies of high-dimensional imaging markers are now underway. It remains to be seen whether this approach will lead to the identification of more genetic loci and how these are related to dementia.

IMPLICATIONS FOR CURRENT AND FUTURE TRIALS AND THE ROLE OF PREDICTION

With high failure rates of large, phase III trials of disease-modifying or arresting drugs, the search of finding successful pharmacological therapies for this detrimental disease is one the most challenging and expensive healthcare issues to date [85–87]. To combat this challenge, the focus has shifted from development of treatment strategies in advanced disease stages toward preventive intervention approaches in asymptomatic states or early disease to delay or prevent the onset of dementia. As such, pharmaceutical companies, policy makers and trialists are looking at population-based studies to inform them on optimal design of preventive trials. Such contributions are first in the form of mapping the potential for prevention given current knowledge of potentially modifiable risk factors and second providing risk models to identify high-risk individuals that would benefit most from an effective intervention and therefore should primarily be targeted for trials. We discuss both aspects here.

Back in 1996, a first report came out hinting towards the potential prevention of Alzheimer's disease and dementia [88]. Over the past decades, this approach was substantiated by extensive evidence on the association of vascular risk factors and the risk of dementia [89–93]. The opportunities of primary prevention through modification of vascular and lifestyle factors has been further fueled by accumulating evidence, including initial observations from the Rotterdam Study, that age-specific incidence of dementia is declining in developed countries [7, 94, 95]. These findings have in part been attributed to a better education in early life, and a healthier lifestyle, including an improvement management of vascular risk. Indeed, recent studies have provided quantitative and convincing evidence that up to a third of all dementia cases may be prevented if modifiable risk factors, such as diabetes, smoking, and physical inactivity, were eliminated [14, 15]. The importance of these observations has recently been underscored and anchored by an international committee of experts, consolidating dementia prevention by means of lifestyle improvement as a global yet ambitious strategy to reduce the burden of this disease globally [30]. Building on evidence forthcoming from these observational studies, several large, randomized controlled trials have been conducted assessing the efficacy of multi-domain lifestyle interventions to prevent cognitive decline in community-dwelling individuals [96–99]. So far, most of these trials have been inconclusive, yet the FINGER trial found evidence that these efforts may be more effective in a high-risk population [96]. Future trials are planned to target these interventions at high-risk individuals in the general population, such as the US-POINTER and SINGER trials [100].

The use of biomarkers is increasingly advocated for purpose of risk stratification in selected, high-risk populations (e.g., memory clinics). These biomarkers include amyloid and tau protein levels assessed by cerebrospinal fluid or positron emission tomography (PET), and rare genetic variants with high individual risk. Yet, such approaches cannot be easily translated to the general population for various reasons. As such, there are currently no established risk prediction models for dementia [101]. In a recent effort, we sought to validate currently proposed risk prediction models, but concluded that beyond age these risk models do not meaningfully contribute to risk prediction of dementia [102].

FUTURE PERSPECTIVES

The size and community scope of the Rotterdam Study necessitate striking a fine balance between the drive to include expensive and burdensome investigations versus feasibility of such investigations in a volunteer population. Other considerations involve the structural shortage of research funding and the advent of big data initiatives like the UK Biobank, German National Cohort and the US Precision Medicine Initiative. The latter encourage seeking a next level of detail in formerly considered large populations of ten to twenty thousand individuals. In such an ever-evolving research field, the choice of phenotypes for the study of dementia in the Rotterdam Study will continue to be driven by earlier observations of risk factors and biological changes in the preclinical disease course and facilitated by technological advancements. Brain MRI has been part of the core protocol since 2005, but more recently functional MRI has been added, and we expect the first results of these efforts shortly. Moreover, addition of arterial spin labelling sequence, with the possibility of including a vasomotor challenge, may add the desired detail to map cerebral hemodynamics in the population, as could more novel measurements like near infrared spectroscopy for (changes in) frontal lobe perfusion, and sidestream dark field imaging

for direct visualization of the capillaries. Contrast enhancement could now add further insight into blood-brain barrier function [103], which is increasingly recognized to play a role in Alzheimer's disease [104]. Other brain imaging techniques, including the use of PET amyloid tracers, are still expensive to apply in large numbers of individuals, but become applicable in smaller studies embedded in the larger design of the Rotterdam Study. Mapping trajectories of amyloid deposition with repeated measures in (initially) healthy individuals is vital to determine the disease course, identify why neuropathology accumulates in many individuals, and why this leads to cognitive disturbances in some, but not others. Beyond brain imaging, the Gothenburg studies have shown that cerebrospinal fluid sampling in forthcoming healthy individuals is safe and feasible [105]. This would not merely benefit etiological and prognostic research of amyloid and tau (for which less invasive means could now suffice), but also allow to determine passage of metabolites through the blood-brain barrier, measure intracranial pressure [106], and perhaps most importantly ready research for the development of novel, yet unidentified markers that can be aptly assessed and validated in previously collected community samples. This preparation is crucial to have long-term follow-up of participants available for rapid validation of proposed prognostic markers for dementia, in CSF as well as plasma and serum. Understanding differences and similarities between measures in CSF and plasma by direct comparison will likely be key to development of useful markers in the latter. Notable candidates for such markers involve angiogenesis [107], lipid transport and metabolism [62], and inflammation [108]. Genetic studies invariably implicate immune response in the onset of Alzheimer's disease [62, 67]. We have previously found support for a role of inflammation in the Rotterdam Study [109], yet repeated measurements of ideally more specific cytokines are needed. Other candidate markers may in the coming years be identified from ongoing collaborative efforts that map metabolomic changes in the periphery [110].

CONCLUSION

In conclusion, for nearly 30 years now the Rotterdam Study has contributed greatly to the understanding of dementia, in terms of incidence, risk factors, pathobiology, and prognosis. It achieved its success through exploring novel underlying pathologies, pioneering various emerging technologies in a population-based setting, and maintaining a methodologically sound basis. At the same time, the Rotterdam Study has been a key contributor to various worldwide collaborations. In coming years, we expect the Rotterdam Study to continue its contribution within the vast landscape of dementia research.

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The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831. All participants provided written informed consent to

participate in the study and to have their information obtained from treating physicians.

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Religious Orders Study and Rush Memory and Aging Project

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Abstract.
Background: The Religious Orders Study and Rush Memory and Aging Project are both ongoing longitudinal clinical-pathologic cohort studies of aging and Alzheimer's disease (AD).
Objectives: To summarize progress over the past five years and its implications for understanding neurodegenerative diseases.
Methods: Participants in both studies are older adults who enroll without dementia and agree to detailed longitudinal clinical evaluations and organ donation. The last review summarized findings through the end of 2011. Here we summarize progress and study findings over the past five years and discuss new directions for how these studies can inform on aging and AD in the future.
Results: We summarize 1) findings on the relation of neurobiology to clinical AD; 2) neurobiologic pathways linking risk factors to clinical AD; 3) non-cognitive AD phenotypes including motor function and decision making; 4) the development of a novel drug discovery platform.
Conclusion: Complexity at multiple levels needs to be understood and overcome to develop effective treatments and preventions for cognitive decline and AD dementia.

Keywords: Alzheimer's disease, cognitive decline, decision making, dementia, drug discovery, epidemiology, motor function, neuropathology, omics

INTRODUCTION

For more than a century, careful clinical characterization followed by examination of neural tissues after death has been an important approach for identifying the neuropathologic determinants of dementia [1, 2]. The vast majority of older adults studied in clinical-pathologic studies are recruited at tertiary care dementia centers [3, 4]. In the early 1990 s, community-based cohort studies of aging and dementia started obtaining autopsies. This is important as autopsies from community participants differ from autopsies of individuals evaluated at dementia centers [5]. The first community-based studies were the Nun Study, the Honolulu Asia Aging Study (HAAS), and the Hisayama Study [6–8]. Participants in the Nun Study were over age 75 at entry, and all agreed to organ donation; however, it was not explicitly designed as a study of risk factors for incident AD dementia. Both HAAS and the Hisayama

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Study were population-based studies of risk factors for AD dementia. Both added organ donation for the relatively small number that agreed. Later, other community-based studies in the USA and Europe started to obtain autopsies [9].

The Religious Orders Study (ROS) and Rush Memory and Aging Project (MAP) began in 1994 and 1997, respectively (together referred to as ROSMAP). They are both cohort studies of risk factors for cognitive decline and incident AD dementia, and other health outcomes. They share all essential attributes of analytic cohort studies, and both require an agreement for organ donation as a condition of study entry. The last review of these studies summarized findings through the end of 2011 [10, 11]. Here, we describe the current datasets, summarize progress and study findings with emphasis on results from 2012 through 2017, and contextualize the findings with the other advances in the field. As ROSMAP serves as a resource for the aging and dementia research community, we hope the review will orient potential users of the resource to the wealth of data and findings which can be leveraged for future studies.

MATERIALS

Participants

ROS started in 1994 and enrolls nuns, priests, and brothers from across the US. MAP started in 1997 and enrolls lay persons from across northeastern Illinois. Evaluations are annual and all participants in both cohorts are organ donors. This includes brain, spinal cord, nerve, and muscle for those autopsied at Rush (Illinois, southeastern Wisconsin, and northwestern Indiana), and brain only for those autopsied elsewhere (California, central Illinois, central Indiana, Iowa, Kentucky, Louisiana, Maryland, Minnesota, Missouri, New York, Ohio, Pennsylvania, Tennessee, Texas, Washington DC, central and western Wisconsin). All MAP and a few hundred ROS donate blood annually. A large common core of data is shared by both studies allowing efficient merging of data. The two studies support additional sub-studies that address a wide range of other aspects of aging. Many sub-studies are restricted to MAP as nearly all participants are within driving distance of Rush and it is easier for staff to assess them more frequently. The parent studies and sub-studies were all approved by an Institutional Review Board of Rush University Medical Center and all participants signed an informed consent, Anatomical Gift Act, and a repository consent to share data and biospecimens.

Through December 31, 2017, the studies enrolled 3,414 persons of whom 72.6% are female, 88.2% are non-Latino White, 6.3% are African American, 5.5% are Latino (including African American Latinos), and the remainder are other racial groups. Their mean age was 78.3 years and education 16.9 years, and blood was collected from 94.3%. There have been 1,232 cases of incident mild cognitive impairment (MCI) and 764 cases of incident dementia, and only 7.8% have withdrawn. There have been 1,717 deaths and 1,506 (87.7%) brain autopsies and 834 spinal cord, nerve, and muscle autopsies. Of those autopsied, 67.2% are female, 94.8% are non-Latino White, and the remainder were members of other racial groups. Their mean age was 89.1 years and mean education is 16.9 years. Of those autopsied, 31.0% were without cognitive impairment, 23.0% had MCI, 41.4% had AD dementia with or without another condition, and the remainder had another cause of dementia.

The layers of data now available (or currently being generated) in one or both cohorts are illustrated in Fig. 1. These are documented in the Rush Alzheimer's Disease Center Resource Sharing Hub (http://www.radc.rush.edu). The Hub also includes all information and links required to request data and biospecimens, including downloadable data use and material transfer agreements. The Hub automatically updates with ongoing data collection and is actively expanded when new data is available for sharing.

Potential risk factors

Both studies collect a wide range of exposure data that includes genomic, experiential, psychological, and medical risk factors [12–46]. This includes continuous daily recordings of physical activity with an omnidirectional accelerometer [47]. From these recordings quantitative metrics of physical activity, sleep and circadian rhythms are extracted [48]. We administer the Food Frequency Questionnaire [49]. Genome-wide data has been generated [50], and we recently generated whole genome sequencing.

Multi-level omics

Several additional layers of brain and blood molecular genomics were generated. Data generated from the dorsolateral prefrontal cortex, include DNA methylation, H3K9Ac, miRNA, and RNAseq. We are currently generating 5hC methylation, another

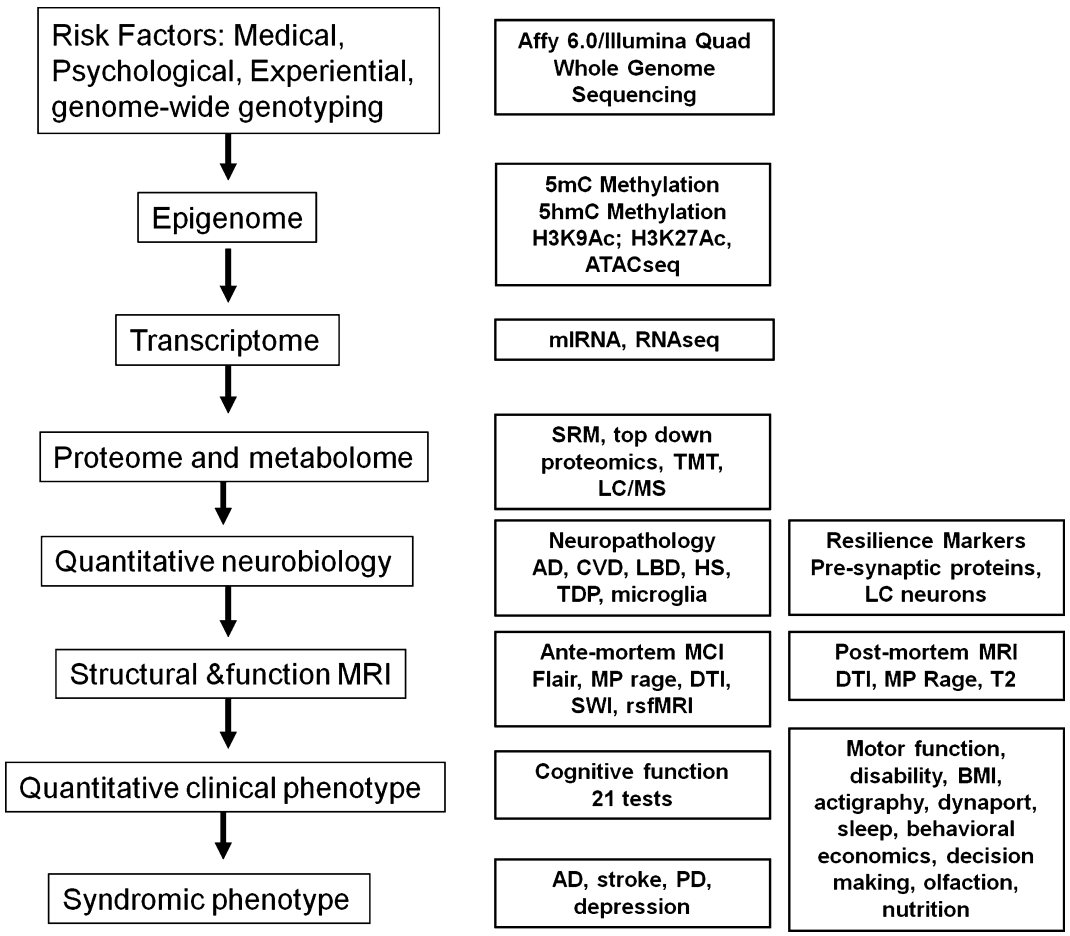

Fig. 1. Multi-layered omics, neuropathologic, and clinical data in ROSMAP.

histone mark, proteomics and metabolomics from the same region, plus RNAseq and DNA methylation from other brain regions. Proteomic and metabolomic data is also being generated on blood, DNA methylation from CD4+ lymphocytes, and RNAseq from monocytes. Finally, we are in the process of establishing 50 iPSC lines from participants.

Neuropathologic and neurobiologic traits

A wide range of neuropathologic traits are generated. These include quantitative measures of AD pathology by histochemistry and immunohistochemistry, and Braak Stage, NIA-Reagan, and NIA-AA pathologic criteria for AD [51–55]. Other measures include macro- and microscopic infarcts, athero- and arteriolarsclerosis, amyloid angiopathy, Lewy bodies, TDP-43, hippocampal sclerosis, and (on subsets) activated microglia and white matter pallor. Arteriolar sclerosis as well as AD pathology and Lewy bodies are also recorded in the spinal cord [56–61]. This is complemented by measures of resilience including presynaptic proteins and neuron density [62]. We also are generating data on targeted proteomics.

Structural and functional neuroimaging

Antemortem 3D MPRAGE, diffusion weighted imaging, 2D fast spin echo, 2D FLAIR, QSM, and resting state functional MRI is done on a subset of participants [63]. We also perform *ex vivo* imaging in many cases both fresh and fixed [64, 65].

Quantitative clinical phenotypes

Twenty-one cognitive performance tests with 19 in common, 17 of which are summarized as measures of global cognition, and scores of episodic memory, semantic memory, working memory, perceptual speed, and visuospatial ability [15, 66–68].

Parkinsonian signs summarized as a continuous measure of parkinsonism, and domains of gait, bradykinesia, rigidity, and resting and/or postural tremor, and a categorical measure of parkinsonism [69–72]. Other motor performance tests include quantitative measures of upper and lower limb performance [42, 43, 73–77]. Since 2012, annual gait testing now includes a body sensor with a triaxial accelerometer with three gyroscopes [78–82].

We also have studies of behavioral economics, decision making, and related behaviors in MAP. This includes measures of risk aversion and temporal discounting, health and financial decision making, health and financial literacy, and susceptibility to scams and fraud victimization, and related psychological measures (e.g., purpose in life) [40, 83–86].

Syndromic clinical phenotypes

Clinical diagnosis of dementia, especially AD dementia, and MCI are documented [87, 88]. We also make diagnoses of stroke and vascular cognitive impairment, Parkinson's disease (PD), and depression [89–91]. Other diagnoses are made by history and examination of medications. Diagnoses are rendered annually and a final diagnosis prior to death is generated after review of all data blinded to neuropathology. For some participants, we have linkages to Medicare data.

RESULTS

Several themes have dominated our work over the past six years. One is the relation of neuropathologic and resilience indices to cognitive decline, MCI, and AD dementia. Second are the neurobiologic pathways linking risk factors to cognitive decline, MCI and AD dementia. A summary of these associations are illustrated in Fig. 2. Third is a comparable portfolio centering on motor structure and function including parkinsonism, and a fourth on behavioral- neuroeconomics and decision making. Finally, we describe our emerging novel drug discovery pipeline.

Due to the large number of annual assessments over so many years, we calculated the attributable risk of death due to incident AD dementia [92]. Time from incident AD dementia to death was less than 4 years with a hazard ratio of more than 4. Upweighted to the US population resulted in an estimated 500,000

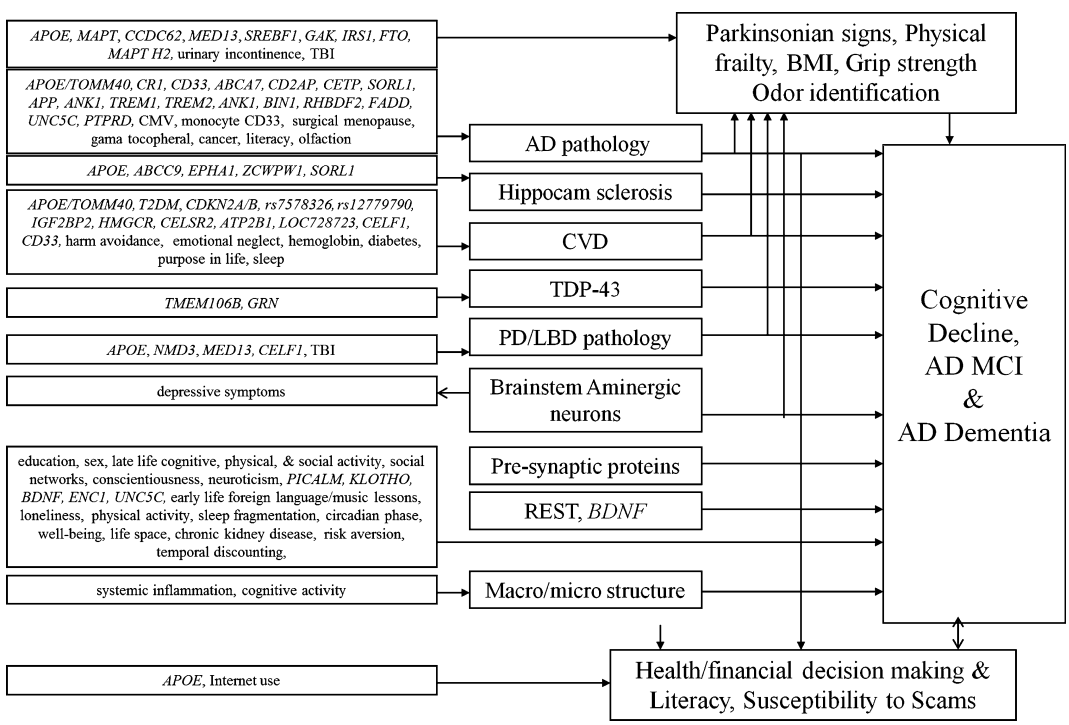

Fig. 2. Neurobiologic pathways linking risk factors to AD clinical phenotypes.

deaths attributable to incident AD dementia in 2010 putting it on par with cancer and heart disease.

Relation of neuropathology and resilience indices to cognitive decline, MCI, and AD dementia

We demonstrated that mixed pathologies were the most common cause of AD dementia with AD pathology including amyloid angiopathy, several indices of cerebrovascular disease, including macro- and microinfarcts, atherosclerosis, arteriolarsclerosis, Lewy body disease, TDP-43, and hippocampal sclerosis all having additive effects on the odds of AD dementia [93–102]. Some pathologies, such as atrophy and white matter changes, were assessed with *in vivo* or *ex vivo* imaging and were also separately related to dementia [103–110].

A dozen years ago we first reported that pathologic AD was present in about a third of older persons without dementia or MCI and that it was related to episodic memory [55]. In a follow-up we showed that neocortical amyloid-β, mesial temporal PHF-tau tangles, and macroscopic infarctions were all related to episodic memory and amyloid-β related to working memory in persons without cognitive impairment [111]. We recently extended this work showing that TDP and hippocampal sclerosis are associated with cognitive impairment in persons without pathologic AD, similar to earlier findings with AD pathology, Lewy bodies, and infarcts [101].

An important feature of the study design, the repeated measures of cognition, permits one to examine the relation of pathologies to the trajectory of cognitive decline over up to a quarter century prior to death. Using several approaches including latent variable models, change point models, Markov chain models, and sigmoidal models we find that the effects of neuropathology on cognitive decline emerge many years prior to death [112–123]. These data further illustrate the continuum of the clinical AD phenotype that seamlessly evolves from normality, to minimal then mild cognitive impairment, and eventually to dementia. Further, they show that effects of pathology, including hippocampal volume, measured at death are related to cognitive changes many years prior to death, including in persons who died without dementia [120, 124].

Using a Markov chain model, we illustrated the effects of multiple pathologies on the "horserace" between dementia and death [121]. Without any pathology there is nearly a 20% likelihood of cognitive impairment prior to death. By contrast, with AD, infarcts, and Lewy body disease, the risk triples to nearly 60%. We recently reported nearly 250 different unique combinations of pathologies accounting for cognitive decline in just over 1000 persons [122]. The most common, pathologic AD alone was less than 10%. Nearly 100 people had a combination that was not present in any other person. Further, the magnitude of the effect of each pathology on cognitive decline varied widely depending on the specific combination present.

Interestingly, we found that when we link common neuropathologies to cognitive decline, we explain less than half of the person specific differences in slopes [124, 125]. This likely results from several factors. First, neuropathologies are neither measured perfectly nor completely, and downstream effects of measured pathologies are only captured on a subset of participants [105–108]. In addition, we are not measuring all of the brain pathologies known to be associated with the named diseases. For example, we only have soluble pathologies on a small subset of participants [126–131]. Further, it is likely that new associated pathologies will be discovered in the future.

However, another important factor is neural reserve or resilience. We define resilience as a continuous (latent) variable defined as cognitive decline not explained by extant pathologies, i.e., residual cognitive decline [132]. When viewed in this way, every person has some resilience. However, one can be more or less resilient relative to the average person and therefore have a slower or faster rate of residual cognitive decline. We found several genomic and neurobiologic indices of resilience were associated with a slower rate of decline including presynaptic proteins, neuron density, and *BDNF* expression [122, 132–137]. We are also finding genes associated with a faster rate of cognitive decline [138].

Neurobiologic pathways linking risk factor to cognitive decline, MCI, and AD dementia

We first examine change in cognition and show that cognitive decline in African Americans and Latinos in our cohorts was similar to whites [139–141]. We also conducted a series of change point models in persons who developed AD dementia and showed that change in cognition began years prior to onset of AD dementia, and among persons who developed MCI, change in cognition began long before diagnosis [142]. Next, we summarize four sets of risk factor associations.

Genomic risk factors

We examined the effects of the *TOMM40* haplotypes to cognitive decline, incident AD, and neuropathology [143–146]. Due to its strong linkage disequilibrium with *APOE*, we restricted one analysis of Caucasians to persons with *APOE* ε3/3 genotype and found that both *'523-L* and *'523-S/S* S/S poly-T genotype were related to faster cognitive decline, especially episodic memory, a finding similar to *APOE4* [146]. In another study we examined racial differences and found that among Caucasians nearly all *APOE4* carriers had *'523-L* whereas less than half of the African Americans had this haplotype [144]. In African Americans, the *ε4-'523-L* haplotype had stronger effect on risk of AD dementia than other *APOE4-'523* haplotypes. This contrasts with the effects of *APOE4* among African Americans which is much weaker than in Caucasians [147]. Interestingly, the effect of the *'523-L* poly-T genotype was attenuated and no-longer significant controlling for AD and other neuropathologies, again similar to what we found for *APOE4* [148–151]. By contrast, *'523-S/S* S/S association with unchanged in analyses with neuropathologies suggesting that the two haplotypes work via different pathologic mechanisms.

We also examined in more detail are several single nucleotide polymorphisms (SNP) that emerged from prior genome wide association studies (GWAS) [152]. We found that *CR1, SORL1*, and *CD33* were all associated with cognitive decline, AD pathology and amyloid angiopathy [153–159]. *CD33* also modulated *TREM2* in monocytes [156]. When examining all genomic variants from prior GWAS, we found some were associated with AD pathology but as a result of mixed pathologies and resilience, others were associated with co-morbid pathologies (i.e., *ZCWPW, SORL1*, and *APOE* with hippocampal sclerosis, *CELF1* with Lewy bodies and microinfarcts, and *ABCA7* with macroinfarcts), and some were not associated with any pathology [160]. We used DNA methylation to delve further into the known genomic variants and found associations between DNA methylation in several AD genes [161–163].

We found other genomic variants associated with cognitive decline and AD, and some associated with other pathologies or with no pathologies [164–172]. We also used GWAS to identify genomic variants associated with resilience and found two genes, *ENC1* and *UNCSC,* that also showed evidence with DNA methylation and expression [162]. Interestingly, we also found that *UNCSC* was associated with amyloid angiopathy [173]. We did not find evidence of an association of the fragile X permutation expansion with cognition [174]. We also conducted GWAS for neuropathologic traits [175, 176]. Finally, we identified a variant in *TMEM106B* and expression of *GRN* associated with TDP-43 [177].

Experiential risk factors

Using change-point models, we found that education was associated with better cognition, a slower rate of cognitive decline and a delayed change point but a more rapid rate of decline after the change point [142]. Further, life-time cognitive activities as well as foreign language and music instruction were associated with a slower rate of cognitive decline including among Latinos [178, 179]. To address the potential for reverse causality, we used a cross-lagged model to show that cognitive activities initially predicts cognitive decline but as cognition becomes poor, cognition predicts decline in late-life activity [180]. Further, late-life cognitive activity was not related to common neuropathologies [181]. However, it was related to brain microstructure by neuroimaging, which partially mediated the association of cognitive activity with level of cognition [182]. We also found that total daily physical activity measured by actigraphy was associated with risk of AD dementia [183] and negative social interactions were associated with incident cognitive impairment [184].

We also found that both the DASH and Mediterranean diets were associated with a slower rate of cognitive decline [185]. We created the MIND diet which combines elements of the other two diets and found a stronger association with cognitive decline and AD dementia risk [186, 187]. Green leafy vegetables and seafood were individually associated with cognitive decline, the latter driven by consumption of foods high in long-chain omega-3 fatty acids [188–190]. Interestingly, in matched plasma and brain samples, we found lower levels of oleic acid isomers and omega-3 and omega-6 fatty acids as well as oleic acid in AD plasma [191]. By contrast, we only found lower docosahexaenoic acid (DHA) in brain. Interestingly, we found that fish consumption was associated with measures of AD pathology [192]. This is one of very few non-genomic factors that we found directly associated with measures of AD pathology. The finding was restricted to those with *APOE4*, but that could result from greater power. Higher α-linolenic acid (18:3 n-3) was associated with fewer cerebral macroinfarctions. Finally, we found that γ-tocopherol concentrations were associated with less AD pathology [193].

Psychological risk factors

We previously showed that depressive symptoms were associated with risk of AD dementia and did not change as AD dementia developed suggesting that the association is not reverse causality [13, 35]. Recently, we controlled for neuropathology and showed that it did not influence the association, nor were depressive symptoms a consequence of typical pathologies that cause dementia [194, 195]. Interestingly, lower density of dopamine neurons in the ventral tegmental area was associated with more depressive symptoms [196]. We previously found that rate of cognitive decline increases several fold about four years prior to death, a concept referred to as terminal decline [197, 198]. We found that conscientiousness was related to a slower rate of terminal decline and that this trait attenuated the association of Lewy bodies with terminal decline [199].

We found that cognitive decline was associated with several aspects of reduced well-being, or eudaimonic happiness [200]. One aspect of well-being, purpose in life, was associated with risk of AD dementia and modified the relation, of pathology to cognitive decline [201]. It was also associated with reduced odds of cerebral infarctions [202], as well as with reduced hospitalization [203]. By contrast, childhood emotional neglect and harm avoidance were both associated with increased odds of cerebral infarction [204, 205]. Further, neuroticism modified the association of vision with cognition [206]. Finally, loneliness was associated with AD risk and cognitive decline, but not with neuropathologies [30]; and we identified numerous genes in the amygdala and the dorsolateral prefrontal cortex related to loneliness [207, 208].

Medical factors

We first examined cerebrovascular disease factors. Lower body mass index (BMI) was related to cognitive decline in both African Americans and whites [209]. Also, lower hemoglobin was related to macroscopic infarcts [210]. We did not find associations of antiphospholipid antibodies to any measure of cerebrovascular disease or of genetic variants associated with homocysteine to be associated with any pathology [211, 212]. Diabetes was associated with subcortical macroscopic infarcts [213]. Interestingly, we found that insulin resistance in brain was related to measures of AD pathology [214]. We found that initiation of anticholinergic medicine had a negative impact on the slope of cognitive decline [215]. Also, antibodies to cytomegalovirus were related to cognitive decline and AD dementia in both African Americans and whites and was associated with measures of AD pathology [216, 217]. Better odor identification on a smell test was positively associated with cognition, and worse scores were associated with loneliness and depressive symptoms [218]. When we examine the anterior olfactory nucleus, we find co-localization of amyloid-β, PHF-1, and cCaspase-6, and the level of PHF-1 and caspase-6 were positively correlated [219]. In two other papers we found that surgical menopause was related to cognitive decline and neurofibrillary tangles, and history of cancer was associated with a lower likelihood of AD dementia and PHF-tau tangles [220, 221]. Finally, in an *in vivo* imaging study restricted to persons without dementia, we found that c-reactive protein and tumor necrosis factor-alpha were associated with cognition and brain microstructure [222]

Using data generated with the accelerometer, we developed a metric of rest-activity fragmentation as a proxy for sleep fragmentation [48, 223]. This measure was related to cognition and incident AD dementia, as well as lower cortical gray matter volume in the inferior frontal gyrus pars orbitalis and lateral orbitofrontal cortex [224, 225]. It modified the relation of *APOE4* to measures of AD pathology and was directly associated with measures of cerebrovascular disease [226, 227]. The same data was used to generate circadian rhythms. We found inter-daily variability associated with the metabolic syndrome [228]. Further, we investigated the influence of several clock genes, using genomic, epigenomic, and transcriptomic data on circadian and seasonal rhythms [229–233]. Separately, we found that sleep fragmentation and circadian rhythm disruption were related to neuron counts in the ventrolateral preoptic/intermediate nucleus of the hypothalamus, and the suprachiasmatic nucleus [229].

Risk factors, neuropathology, and motor structure and function

Parkinsonism was progressive and associated with adverse health outcomes [183, 230–233]. Changes in motor structure and function were strongly correlated with changes in cognition and both related to the same neuropathologies. Parkinsonism was associated with risk of death, MCI, and AD dementia, and common brain pathologies were related to parkinsonian signs, and progression of physical frailty, respiratory function, and cognitive decline [234–239]. Further, neurons in the locus coeruleus were related to parkin-

sonian signs and to cognition [134, 240]. Thus, it was not surprising that many risk factors for cognitive decline and AD dementia are also risk factors for motor outcomes including physical and social activity, social isolation, neuroticism, harm avoidance, extraversion, and antihypertensive medications [232, 233, 241–244]. By contrast, traumatic brain injury was related to progression of parkinsonism and PD pathology but not change in cognition or AD pathology [245]. Sleep, was also associated with motor outcomes in both African Americans and Caucasians, and with PD pathology [246–248]. We also conducted a candidate SNP analysis examining PD risk alleles with a variety of motor clinical and pathologic phenotypes [249]. We are just beginning to explore the spinal cord examining the distribution of α-synuclein, atherosclerosis, white matter pallor, and their association with brain pathology and motor function [259–261]. Recently, we added a body sensor, a triaxial accelerometer with 3 gyroscopes, which participant's wear on a belt, which continuously records 3 acceleration and 3 angular velocity signals during annual gait testing. We examined the metrics derived from these recording during several gait and balance tests and their relation to IADL, parkinsonism, and physical activity [78–82]. Finally, using resting state fMRI we interrogated connectivity in relation to chronic musculoskeletal pain [250]. In a separate study, we found that physical activity modified the relation of white matter hyperintensities with motor function [251].

Behavioral- and neuro-economics and decision making

We first examined health and financial decision making. We found both associated with risk of death, incident MCI and AD, and cognitive decline among persons without dementia. Further, several factors help maintain decision making, including literacy and access to resources (e.g., internet use) [252–257]. Next, we examined health and financial literacy. These also are associated with cognition, MCI, functional status, mental health, health promoting behaviors, and *APOE4* [258–260]. Literacy is both a consequence of cognitive decline and a predictor of future cognitive decline and incident AD dementia [261–264]. Interestingly, AD pathology was associated with literacy controlling for cognition [264]. Among persons without dementia, we found that higher diffusion anisotropy was associated with better financial literacy, especially tracts connecting right hemisphere temporal-parietal brain regions [265]. Financial literacy was also associated with greater functional connectivity between the posterior cingulate cortex and the right ventromedial prefrontal cortex, the left postcentral gyrus, and the right precuneus, and negatively associated with functional connectivity with left caudate [266]. Greater temporal discounting was associated with increased risk of death, cognition, and cognitive decline [267–269]. Discounting also was positively associated with functional connectivity to the right middle temporal regions and ventromedial prefrontal cortex, and negatively associated with parahippocampal and right cerebellar regions [270]. Risk aversion was associated with decision making and cognitive decline [269, 271]. Using a seed in the anterior cingulate, we found that risk averse persons had greater connectivity to clusters within multiple brain regions (e.g., insula, inferior and orbital, frontal, parahippocampal), and those low in risk aversion had greater connectivity to numerous clusters (e.g., inferior temporal, superior, middle, and medial frontal regions) [272]. We also reported that susceptibility to scams was negatively associated with cognition, well-being, and literacy, MCI, and cognitive decline [85, 273, 274]. There was also an inverse association between overall grey matter and susceptibility to scams [275]. Finally, cognitive decline and over-confidence in one's financial knowledge was associated with fraud victimization [85].

Novel drug and biomarker discovery pipeline

The multilayer omics data are now being used to support a novel drug and biomarker discovery pipeline as part of the Accelerating Medicines Partnership-AD (Fig. 3) [276, 277]. We are still at the early stage of generating omics data, performing the quality control, and developing a basic understanding of relationships with AD quantitative endophenotypes with epigenomic and transcriptomic data [278–286]. We are just beginning to examine relations of omic between brain and blood and brain and neuroimaging as part of our nascent biomarker discovery protocol [287, 288]. This will be complemented in the future with multiple layers of blood omics that can be related to antemortem imaging and brain omics. Further, we are still refining our *ex vivo* validation approaches [289, 290]. A forward-looking framework has been developed and we will focus on neural reserve or resilience as a high value target [291, 292]. We identified one high value module

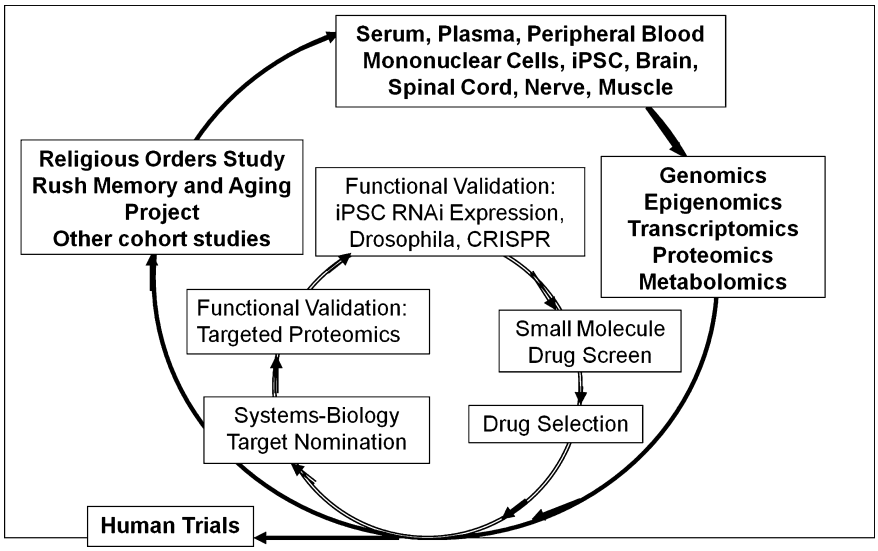

Fig. 3. Cohort studies generating ante- and postmortem biospecimens which are used to generate multi-layered omics data. These data feed a systems biology computation pipeline for therapeutic target nomination. There are two stages of functional validation, one with targeted proteomics using brain tissue from the same cases and the other with a variety of high throughput *ex vivo* models. High value targets will then move to small molecule drug screen and eventual drug selection.

that nominates genes/proteins that drive resilience and others that drive amyloid-β [138]. The latter have been validated in an *ex vivo* model system. Finally, as we look forward to better clinical trial designs, we have been refining our cognitive phenotype that would best serve as a clinical trial outcome [293–295].

Resource sharing

The ROSMAP investigators are committed to timely data sharing of raw or processed data which can be found on our Resource Sharing Hub with relevant links to the AMP-AD Knowledge Portal [296, 297]. A sample of work done with ROSMAP data including as part of translational studies and consortia is provided to give the community a better sense of the range of work that could be done leveraging the resource [298–350].

DISCUSSION

Ongoing for nearly a quarter of a century, ROSMAP has generated a wealth of data across a range of age-related phenotypes from the same individuals. It has served as a research resource for investigators around the globe who have generated about 400 publications over the past 6 years. The data support some general conclusions. Perhaps the over-arching theme running through the work is complexity. Complexity at multiple levels: 1) continuum of AD, 2) mixed and newly recognized pathologies, 3) neural reserve or resilience, and 4) non-cognitive phenotypes. Complexity has important implications for drug discovery.

Continuum of AD

Findings illustrate that cognitive decline as part of the AD dementia syndrome begins years or decades prior to AD dementia onset and MCI onset. We found that AD and other pathologies are common in persons without dementia and without MCI. Further, AD pathology is associated with change in cognition a decade or more prior to death, and that these associations occur long before dementia onset. These data complement data from other clinical-pathologic studies illustrating that AD pathology is present in persons without dementia [351–353]. However, there is much less information from other studies linking pathology to trajectories of cognitive change [354–357]. These data are complemented by clinical-imaging studies with amyloid and subsequently tau PET data [358–362]. More recently, amyloid and tau PET done on younger cohorts are finding that these pathologies appear to accumulate years prior to onset of overt cognitive impairment [363–366]. Together these data provide strong support for a new framework being

proposed to capture the continuum of AD that allows a diagnosis of AD to be made in the absence of cognitive symptoms [367]. The framework is based largely on a recently proposed A/T/N classification scheme [368].

Mixed and newly recognized pathologies

We find that the clinical syndrome AD dementia cognitive impairment and dementia is a complex process that results from the additive and interactive effects of numerous pathologies. This is consistent with the results of several prior clinical-pathologic studies that have examined this issue [4, 369–377]. Similar data are emerging from neuroimaging studies as well [378–380]. At this point, we have documented 9 pathologies on more than 1000 brains and find nearly 250 combinations. Besides AD pathology, this includes TDP-43/hippocampal sclerosis, several measures of macro- and micro-vascular disease. We also have other pathologies on a subset of participants, including some measured with *ex vivo* imaging, which will further increase the number of combinations.

Neural reserve or resilience

There are several approaches to the concept of neural reserve or resilience [381–384]. Many researchers limit the concept of reserve or resilience to having a unidirectional beneficial effect. We take a complementary approach that assumes all cognitive systems have some reserve; however, some have more reserve and others less [385]. Persons with more reserve have a slower rate of cognitive decline and lower AD dementia risk, and those with less reserve have a faster rate of cognitive decline and higher AD dementia risk. We have found many risk factors associated with more (e.g., cognitive activity, purpose in life) or less (e.g., neuroticism, loneliness) reserve, and some biologic factors associated with more reserve (e.g., *BDNF* expression, neuron density, presynaptic proteins), and network modules with genes associated with more and others with less reserve [292]. Several other groups have reported on the ability of factors to buffer or augment the impact of pathology on cognition [386–392]. Similar findings have been reported with neuroimaging and CSF biomarkers of AD pathology [361, 392–398]. Functional imaging approaches are also being employed to explore the neural basis of reserve [399, 400]. Finally, similar to our findings of neurons and presynaptic proteins, several groups have reported other structural indices that underlie reserve [401–404].

Non-cognitive phenotypes: Motor function and decision making

Our work is congruent with work by other groups that a wide range of motor phenotypes are related to cognitive decline and to AD [405–410]. In addition, like others we find that cognitive function and many risk factors for AD are also related to change in motor structure and function [411, 412]. Our work extends these findings by showing that simultaneous change in cognitive and motor decline is highly correlated [239]. Few studies address whether cognitive or motor decline begins earlier [413]. The idea that both late-life cognitive and motor impairment may share a common neurobiology is supported by our postmortem results and work by others [404, 414–418]. Our studies extend these findings by showing that AD and other pathologies are related to level as well as progressive decline of several motor phenotypes [234, 236, 238, 239]. Together the clinical and postmortem findings are consistent with accumulating evidence that both cognition and motor function may rely on similar underlying neural systems essential for planning and monitoring goal–directed behavior and both may be affected by AD and other common brain pathologies [367, 419–422].

We also showed that cognition, AD, and other pathologies negatively impact health and financial decision making [254–258, 269, 270]. This work is consistent with prior studies that have reported impaired decision making among persons with overt cognitive syndromes [423–427] and some small studies of non-demented persons [428–432] but extends prior work by showing that impaired decision making among cognitively intact persons is in fact a consequence of preclinical cognitive decline. Few studies have examined the association of decision making and related behaviors with subsequent cognitive or other health outcomes [423, 428, 431]. Our work suggests that decision making and related behaviors predict several adverse health outcomes including incident AD, incident mild cognitive impairment and mortality [252, 262–265, 268]. Moreover, whereas some prior studies have examined the neural underpinnings of select aspects decision making in older persons using neuroimaging approaches [433–439], we expanded this work by examining multiple aspects of decision making using a variety of imaging approaches [265–274] Finally, we found that age-

related changes in decision making are associated with common neuropathologies such as AD pathology [264, 265]. Together, findings suggest that impaired decision making is an early manifestation of AD and other neuropathologies and a harbinger of adverse cognitive and other health outcomes.

Implications of complexity for drug discovery

It has been a bleak 15 years in the AD drug discovery space. Other than re-formulations, no new drug has been approved by the Food and Drug Administration (FDA) since 2003. The string of failed studies is long despite the investment of billions of dollars from the public and private sectors, the participation of many tens of thousands of people in clinical trials, and the efforts of thousands of researchers and study staff [440]. There are many reasons for these failures. A recent analysis pointed to complexity, low signal-to-noise, and recruitment/retention [441]. As many people have and others will develop AD dementia, more robust symptomatic treatment is urgently needed. However, symptomatic therapies will not reduce the overall human and economic toll of AD [442]. This can only be accomplished by prevention.

There are currently about 100 drugs in the AD pipeline in the USA with an additional 100 in development in the European Union with participants ranging from those with moderate to severe AD dementia to asymptomatic persons [443, 444]. The therapies are relatively evenly divided into three buckets. The first is small molecules for therapeutic treatment. The other two are disease modifying agents, one of which is small molecules and the other immuno-therapies.

The majority of the disease modifying agents target amyloid and tau. It has been argued that reducing complexity and creating more homogenous populations for clinical trials will improve the signal to noise ratio improving the likelihood of success. Thus, many trials now enrich studies by enrolling those at genetic risk or with a positive amyloid PET [445–447]. Perhaps this will be a successful approach. However, failure will only inform on subpopulations. A drug that fails to slow cognitive decline among persons at genetic risk or those who have amyloid might still work on those not at genetic risk of those who have not yet developed amyloid. Estimates suggest that studies to slow cognitive decline in asymptomatic persons may need to be much longer than is currently being done [448]. Further, requiring multiple spinal taps and/or PET scans likely increases the healthy volunteer effect by excluding people with non-cognitive factors that predict cognitive decline and are associated with AD pathology such as gait disturbance and frailty [235, 237, 239, 449]. We also found that amyloid-β does not predict cognitive decline after controlling for tangles and that amyloid-β and tangles together only account for about 25% of the variance of cognitive decline [118, 125, 450]. Are anti-amyloid studies adequately powered to impact such a small component of the trajectory? In addition, we found that the impact of nine common pathologies, e.g., pathologic AD, on cognitive decline varies widely depending on the presence of other pathologies, many of which are beyond the resolving power of extant biomarkers. Finally, is developing a biomarker for each pathology and a cocktail to treat each pathology really scalable? This could result in multiple cocktails over a long period of time in older persons with aged livers and kidneys, at a cost that is likely beyond what can be paid.

An alternate therapeutic strategy would be to target resilience itself. All physiologic systems have reserve or resilience. In some cases, it is simply an extra organ, e.g., lung, kidney. However, with the brain it is plasticity that allows it to tolerate and/or recover from injury and disease. There is no evolutionary pressure to develop these systems from age related disease. Thus, there are likely few such systems and they are, as we have found, relatively agnostic to specific age-related disease. We are currently using our drug discovery pipeline to find novel targets for reserve. To determine if they are druggable, the field needs to develop and validate an *ex-vivo* model for high throughput drug screens. In other words, we will need to model cognitive decline in a dish.

ADDENDUM

To ensure the review is up to date, we add a list of manuscripts published or accepted for publication since the manuscript was last submitted.

1. Dawe RJ, Leurgans SE, Yang J, Bennett JM, Hausdorff JM, Lim AS, Gaiteri C, Bennett DA, Buchman AS. Association between quantitative gait and balance measures and total daily physical activity in community-dwelling older adults. *Journal of Gerontology: Medical Sciences* 2018;73:636-642.
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12. Buchman AS, Dawe RJ, Lei Y, Lim A, Wilson RS, Schneider JA, Bennett DA. Brain pathology is related to total daily activity in older adults. *Neurology.* 2018;90:e1911-e1919. doi: 10.1212/WNL.0000000000005552.
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is related to cognitive decline, independent of small vessel disease in older adults. *Neurobiology of Aging.* Pending revisions.
20. De Jager PL, Ma Y, McCabe C, Xu J, Vardarajan BN, Felsky D, Klein HU, White CC, Peters M, Lodgson B, Nejad P, Tang A, Mangravite L, Yu L, Gaiteri C, Mostafavi S, Schneider JA, Bennett DA. A multi-omic atlas of the human frontal cortex for aging and Alzheimer's disease research. *Scientific Data.* In press.
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Prevention Matters: Time for Global Action and Effective Implementation

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Abstract. During the last few years, dementia prevention based on modifiable lifestyle factors has gained increasing attention. Cohort studies with follow-ups extending up to decades have identified several risk and protective factors, and very recently new randomized controlled trials with multidomain approach have provided promising evidence by showing that modifying simultaneously several risk factors, it is possible to maintain and improve cognitive capacity among older at-risk persons. Several lifestyle-based multidomain trials are under preparation or ongoing and to facilitate international collaboration and effective worldwide dementia prevention, the World Wide FINGERS interdisciplinary network (http://wwfingers.com) was recently initiated. Additionally, several new implementation projects are taking the first steps from trial setting to real-life implementation of a dementia prevention program. This paper highlights the recent perspectives from the field of Alzheimer's disease and reflects the implications and importance of current achievements. Finally, predictions for the future work especially in terms of global collaboration and implementation will be discussed.

Keywords: Dementia, implementation, intervention, prevention, risk reduction

INTRODUCTION

New predictions of the dramatic increase of dementia and Alzheimer's disease (AD) rates worldwide are alarming [1]. There are currently no cure or disease modifying drugs available and recent drug trials have shown mainly negative results. Consequently, prevention has received increasing attention and has been highlighted as the key element in managing the dementia epidemic. During the last two decades, large prospective cohort studies have provided increasing evidence of risk and protective factors throughout the whole life-course which may contribute to the risk of dementia and AD. Lifestyle matters, since it has been estimated that about one third of AD cases could be attributable to modifiable risk factors [2, 3]. Based on these observational studies, new randomized controlled trials (RCT) have started to test whether changes in these modifiable risk factors could decrease the risk for dementia or slow down the progress of cognitive decline. The first randomized controlled trial, the Finnish Intervention Study to Prevent Cognitive Impairment and Disability (FINGER), showed that modifying simultaneously several risk factors, cognitive capacity of older at-risk adults can be maintained and risk of cognitive decline reduced. Following the success of FINGER, several other countries all over the world

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are now planning FINGER-type interventions. This international collaboration has created a new World Wide FINGERS (WW-FINGERS) network, where large research groups are combining their forces to act against the increasing burden of dementia. Further, several implementation studies around the FINGER results are ongoing and general guidelines to dementia prevention are under preparation. This short overview describes the evidence that led to the FINGER trial, introduces the FINGER study and related ongoing activities, presents new results from implementation activities, and also discusses future directions mainly in terms of implementation.

LONG HISTORY OF OBSERVATIONAL STUDIES

We know today that cognitive impairment, dementia, and AD are multifactorial disorders, and evidence from observational studies shows that genetic, vascular, lifestyle-related, and other risk factors often co-occur in the same person, and interact across the lifespan to determine the overall risk of developing dementia and AD. Several large prospective cohort studies around the world have been able to identify a large amount of lifestyle-related risk and protective factors that have great influence on dementia incidence at a population level. The Finnish Cardiovascular Risk Factors, Aging and Dementia (CAIDE) study, which was started already in 1998, was among the first large population-based studies showing the importance of lifestyle related risk factors present already in midlife for dementia development. The CAIDE study linked midlife cardiovascular risk factors such as high blood pressure and cholesterol [4], smoking [5], physical inactivity [6, 7], alcohol consumption [8], poor diet [9, 10], and psychosocial factors [11–13] to increased risk of dementia and AD later in life. Within the CAIDE project, the CAIDE risk score, a simple method for the prediction of the risk of late-life dementia in people of middle age on the basis of their risk profiles, was developed [14] (Fig. 1).

The CAIDE study is still ongoing and current activities include planning the extended follow-up, CAIDE 85+, which will provide opportunity to assess dementia incidence and risk factors among the oldest old, persons aged 90 and over using the follow-up period extending up to 40 years. The CAIDE study has significantly contributed to the current level of evidence on the modifiable risk factors. The risk factors have been a focus of intensive research in the past years, and currently the evidence is strong regarding many of the risk factors (e.g., midlife hypertension, midlife obesity, smoking, education, lack of physical activity), but still less consistent for some other factors, including depression, stress, and social factors. In addition, it is possible that age at the time of risk assessment modifies association between risk factor and outcome. Different risk factors may have critical time window at different time points and risk factors may change during the disease course (e.g., high blood pressure, obesity, cholesterol, and depression). The planned extended CAIDE follow-up study will provide additional evidence and new insights into life-course perspective on cognitive aging.

Technology and internet-based tools are important ways to carry out today's health education and disease prevention. Recently based on CAIDE risk score, a new CAIDE risk score app was developed [15]. The CAIDE risk score app is the first evidence-based mobile app to predict the risk for dementia. Ongoing development will produce similar easily accessible risk assessment and prevention tools for different age and population groups. For example, the EU-funded project Healthy Aging Through Internet Counselling in the Elderly (HATICE) aims to develop an innovative, interactive internet intervention platform to optimize treatment of cardiovascular disease in the elderly and also to investigate whether cognitive decline can be prevented via internet counselling [16]. In the future, the aim is to make e-health risk assessments tools available around the globe, including in low- and middle-income countries and vulnerable populations.

FROM OBSERVATIONAL STUDIES TO GOLD-STANDARD CLINICAL TRIAL

FINGER [17, 18] is the first randomized controlled trial published showing that intensive lifestyle-based intervention targeting simultaneously to several modifiable risk factors has a beneficial effect on the cognitive capacity of older persons who are at increased risk for cognitive decline [17]. The FINGER trial is a 2-year multi-center RCT carried out in Finland and coordinated by the National Institute for Health and Welfare, Helsinki, and conducted in close collaboration with Universities of Eastern Finland, Oulu and Helsinki (Finland) and Karolinska

Risk factor		Points
Age	<47	0
	47-53	3
	>53	4
Education	>10	0
	7-9	2
	<9	3
Systolic blood pressure	<140 mm Hg	0
	>140 mm Hg	2
Body mass index	<30 kg/m^2	0
	>30 kg/m^2	2
Total cholesterol	<6.5 mmol/L	0
	>6.5 mmol/L	2
Physical activity	Yes	0
	No	2

Total score	Dementia risk
0-5	1.0 % (very small risk)
6-7	1.9 % (small risk)
8-9	4.2 % (slightly increased risk)
10-11	7.4 % (moderately increased risk)
12-15	16.4 % (increased risk)

Fig. 1. CAIDE risk score for the prediction of the risk of late-life dementia in people of middle age based on their risk profiles [14].

Institutet (Sweden). The aim of the study is to test the effect of a multi-domain intervention in delaying cognitive impairment and disability in elderly at risk. FINGER enrolled 1,260 participants aged 60–77 years recruited from previous population-based survey cohorts in 2009. Inclusion criteria were: CAIDE Dementia Risk Score >6 points, indicating the presence of modifiable risk factors; and cognitive performance at the mean level or slightly lower than expected for age. Participants were randomized (1:1) into either the multidomain intervention group or the control group. The intervention included nutritional guidance, physical exercise, cognitive training and social activities, and management of vascular risk factors (Fig. 2). The control group received regular health advice.

Primary outcome after 2 years was cognitive performance measured by a comprehensive neuropsychological test battery (NTB) composite Z score. An extended follow-up (after 5 and 7 years) with an ongoing sustenance intervention aims to evaluate longer-term effects of the intervention on dementia and AD incidence, and secondary and exploratory outcomes including blood-based biomarkers and neuroimaging with MRI and PET. The 2-year intervention was finalized in February 2014. Already published main results showed that after 2 years, the NTB scores in the intervention group improved 25% more than in the control group. For some cognitive domains, including executive functioning and processing speed, the impact of the intervention was even larger [17]. Currently several secondary outcomes are being analyzed.

Most recent publications have shown that multidomain intervention improved important dimensions of quality of life [19]. Further, participants with shorter leukocyte telomere length had more pronounced benefits on cognition following the multidomain lifestyle intervention [20], which indicates that participants with shorter telomere length had more room for lifestyle improvements when they entered the study. Since shorter telomere length is associated with poor cognitive performance and dementia, the FINGER intervention may be especially beneficial among individuals with increased risk. New results also show that intake of several vitamins and minerals decreased in the control group but remained unchanged or increased in the intervention group during the 2 years [21]. The FINGER study is thus the first large RCT showing that it is possible to prevent cognitive decline using a multidomain intervention among older at-risk individuals. The results highlighted the value of the feasible and novel multidomain approach that is effective for several cognitive domains.

WHAT LIES BEHIND THE SUCCESSFUL FINGER INTERVENTION?

FINGER is currently the only largescale intervention study which has provided evidence of the benefits of the multidomain intervention. Now among the main interests are to find out the factors that influenced the success of this intervention. The ongoing analyses will show how shorter-term adherence to the FINGER intervention (overall adherence and per domain adherence) is related to longer-term adherence to healthy lifestyle changes. This will provide essential information about how to facilitate healthy

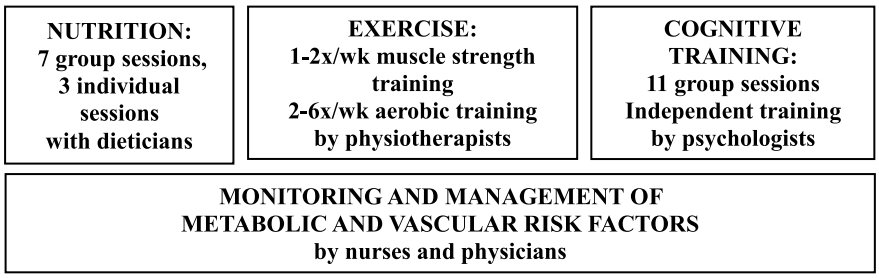

Fig. 2. Intervention components in the FINGER trial.

lifestyle maintenance, and how to optimize the duration of a prevention program. Future analyses will include identifying facilitators and barriers to long-term adherence and identifying effects of adherence level on cognitive decline and dementia incidence. One goal is to tailor the intervention by taking into account how baseline risk level impacts FINGER intervention effects.

One of the main factors behind the success of FINGER intervention was most likely participants' strong commitment for the study and willingness to lifestyle modifications. Detailed feedback regarding intensive intervention was gathered from all participants who received intervention and came at the 2-year follow-up visit ($n = 555$). Feedback was gathered using structured questionnaires and questions focused on the self-reported adherence, common experiences, benefits, and usefulness of the intervention. The results showed that participants perceived intensive 2-year FINGER multidomain lifestyle intervention useful, and most participants intended to continue healthy lifestyle after the intervention. The main feedback from the intensive intervention is summarized in Table 1.

Intensive FINGER intervention lasted for 2 years and required participation in physical and cognitive activities and dietary counselling as well as regular visits to study nurse and physician. The control group got regular health advice from the study nurse and physician. The study design was kept as double-blinded as possible and participants in the FINGER study were not actively told which group they belong to. After the intervention, participants were asked to report their own assumptions of their randomization. Interestingly, almost half of the participants in the intervention group (44%) did not see themselves taking part of intensive intervention, rather instead they thought they only got regular health advice. This gives positive sign that lifestyle modifications used in the FINGER trial were not perceived too stressful and this type of intervention was feasible among older adults at-risk of cognitive decline.

FINGER STUDY AS A MODEL FOR LIFESTYLE INTERVENTION TRIALS IN SEVERAL COUNTRIES

Following the success of FINGER, several other countries are now planning or already starting FINGER-type interventions to test the effect of the multidomain intervention in their own older populations. To facilitate this international collaboration, in July 2017, World Wide FINGERS interdisciplinary network (http://wwfingers.com) was initiated. This new network aims to share experiences, harmonize data, and plan joint international initiatives for the prevention of cognitive impairment and dementia (Fig. 3). The network is led by Professor Miia Kivipelto and the main goal is to generate robust evidence to define effective preventive approaches for various at-risk groups and settings. World Wide Fingers network makes it possible to test sustainable dementia prevention strategies for populations with different geographical, economic, and cultural settings. During the following years, the FINGER multidomain model will be tested in diverse settings in Europe, Singapore, USA, Australia, and China.

FROM RESEARCH TO IMPLEMENTATION: CREATING DEMENTIA PREVENTION TOOLKIT FOR PRIMARY CARE

The FINGER study is considered as a proof of concept trial. It has shown evidence that a multidomain lifestyle intervention results in clear health benefits, and therefore also health care professionals, leaders, and policy makers have shown increasing interest to implement the results into primary care. In close

Table 1
Participants' self-reported adherence, common experiences, perceived benefits and usefulness of the FINGER multidomain intervention (total $n = 555$)

Feedback	Number (%) of participants reporting "YES"
Self-reported adherence	
I nearly always attended the dietary counselling (group sessions)	390 (72)
I nearly always attended the dietary counselling (individual sessions)	438 (83)
I nearly always attended physical activity intervention (group sessions)	316 (60)
I nearly always attended cognitive training sessions	355 (67)
I followed the dietary instructions	360 (67)
I trained in the gym according to given instructions	370 (69)
I did independent physical activity training	305 (57)
I did cognitive training independently according to given instructions	248 (47)
Meaning of social activity	
It was nice to meet other participants	426 (80)
Meeting other participants motivated me to attend the sessions	283 (53)
Feedback from dietary intervention	
Intervention was useful although I knew a lot already	342 (63)
Instructions were good and motivating	504 (94)
Both group and individual sessions were useful	317 (59)
I will follow the dietary instructions after the study	488 (97)
Feedback from physical activity intervention	
I got enough individualized physical activity counselling	466 (97)
My own needs and wishes were taken into account	453 (95)
I got enough instructions to continue training independently	449 (93)
Feedback from cognitive training	
Cognitive training was useful	395 (76)
I got enough instructions to be able to use the computer	367 (73)

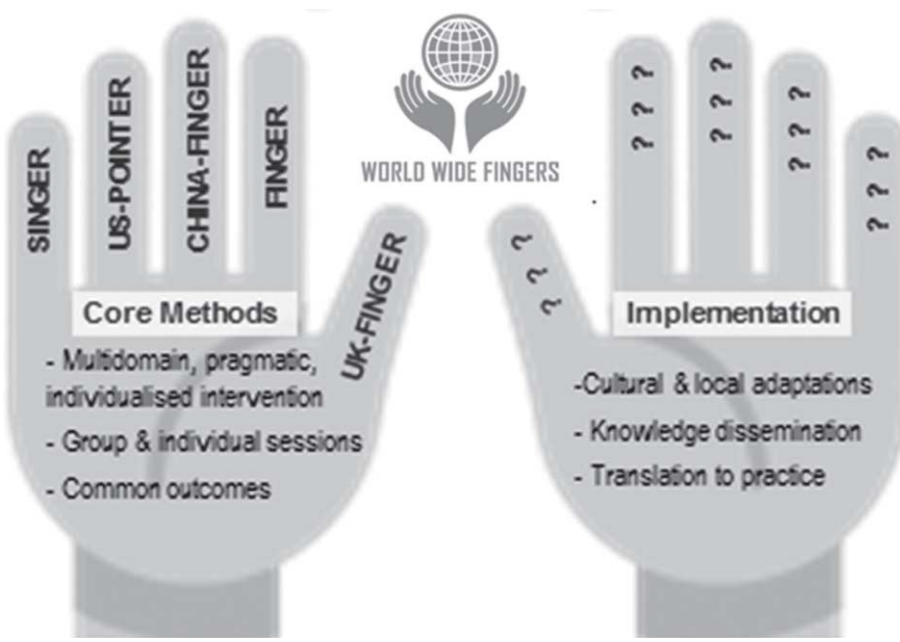

Fig. 3. World Wide FINGERS network aims to share experiences, harmonize data, and plan joint international initiatives for the prevention of cognitive impairment and dementia.

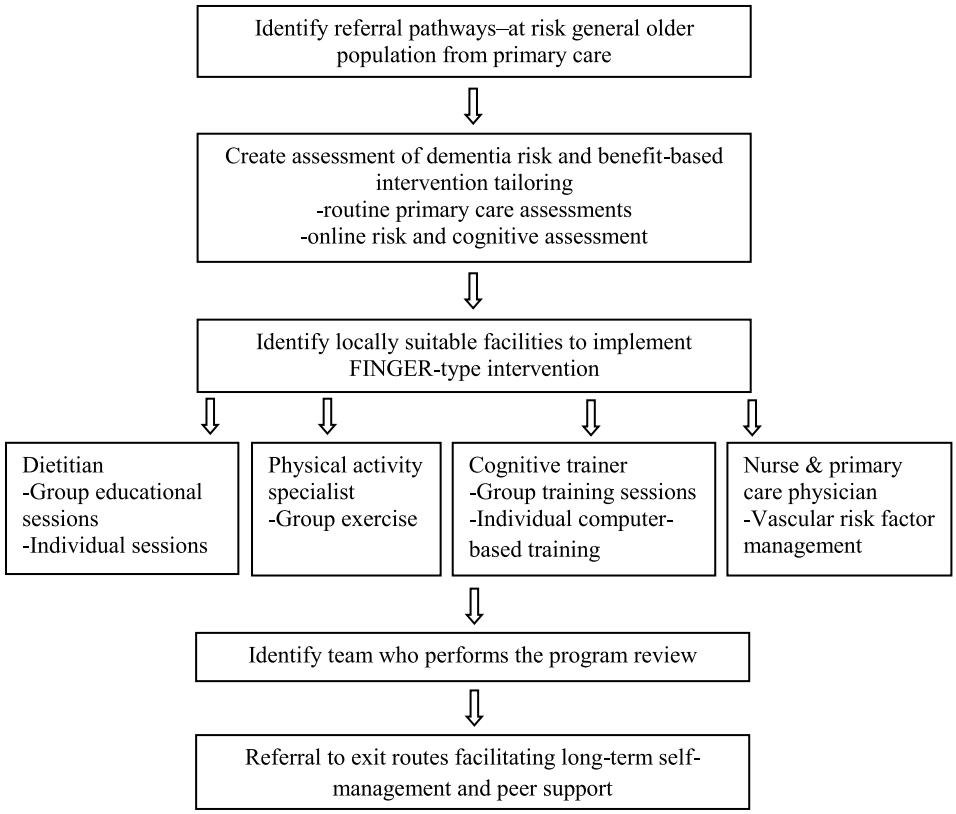

Fig. 4. Proposed operational model for preventing dementia and disability.

collaboration with key stakeholders, including policy makers, health care managers and other health and social care professionals, the first FINGER-based implementation project called MUISTIKKO began in autumn 2016 in Finland. The purpose of the project is to take the first steps from trial setting to real-life implementation of a dementia prevention program. This project provides detailed information on facilitators and barriers to implementation in primary care. Focus will be on communication and education activities, focus groups, and workshops to discuss with multiple stakeholders the practical details needed for future implementation of the FINGER-based operational model in an integrated dementia prevention program. The aim is to establish links between a dementia prevention model and cardiovascular and diabetes prevention models. Close collaboration with stakeholders and health care professionals provides the possibility to gather important information for preparing an implementation toolkit and guidelines for integrated dementia prevention in primary care. The project will lead to proposed operational model (Fig. 4) which will consist of provision of evidence-based means for early identification of at-risk individuals and provision of evidence-based, sustainable intervention strategies for preventing cognitive impairment, dementia, and disability. Also links between the dementia and cardiovascular and diabetes prevention models will be established.

As a result, this first FINGER-based implementation project will provide primary care physicians and nurses with guidelines on how to use available risk assessment tools for making better intervention-related decisions, and for establishing links between the dementia prevention model and cardiovascular and diabetes prevention models. Using information gathered from communication activities and results from focus groups, the aim is to prepare an easy-to-use implementation toolkit and guidelines for integrated dementia prevention in primary care.

SUMMARY AND FUTURE DIRECTIONS

The current evidence suggests that about 30% of all dementia cases are attributable to modifiable lifestyle

related risk factors. Lately, some studies have indeed indicated that age-adjusted prevalence of dementia has been decreasing and the main hypothesis behind the change is that the lifestyle has been improved [22–24]. The FINGER trial has shown that especially when targeting lifestyle intervention simultaneously to several modifiable risk factors and to a high-risk group of older people, the cognitive capacity of older adults could be maintained. Now FINGER serves as a model to other large scale randomized controlled trials all over the world. FINGER intervention is now being replicated in the United States, Europe, Singapore, and Australia and the trials will include populations from a variety of geographical and cultural backgrounds. This worldwide effort, WW-FINGERS, supports a collaborative network of trials and experienced investigators to facilitate harmonization of research methods, and sharing of experiences and data for maximum global scientific impact. During the following years, new results from cohort and intervention studies around the world will provide additional information to improve dementia prevention models. WW-FINGERS network will show how multidomain lifestyle interventions can be replicated worldwide. This global joint effort also provides opportunity for rapid knowledge dissemination and implementation. Lifestyle matters and now it is time for global action and effective implementation.

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Multimodal Neuroimaging in Alzheimer's Disease: Early Diagnosis, Physiopathological Mechanisms, and Impact of Lifestyle

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Abstract. Over the last ten years, we have conducted research in Alzheimer's disease (AD) using multimodal neuroimaging techniques to improve diagnosis, further our understanding of the pathological mechanisms underlying the disease, and support the development of innovative non-pharmacological preventive strategies. Our works emphasized the interest of hippocampal subfield volumetry in early diagnosis and the need for further development in this field including optimization, standardization, and automatization of the techniques. Also, we conducted several studies in cognitively intact at-risk elderly (e.g., subjective cognitive decline patients and APOE4 carriers) to better identify biomarkers associated with increased risk of developing AD. Regarding the physiopathological mechanisms, specific multimodal neuroimaging techniques allowed us to highlight the relevance of diaschisis, the mismatch between neurodegeneration and local Aβ deposition and the regional variation in the mechanisms underlying structural or functional alterations. Further works integrating other biomarkers known to play a role in the physiopathology of AD (tau, TDP-43, inflammation, etc.) in a longitudinal design would be useful to get a comprehensive understanding of their relative role, sequence, and causal relationships. Our works also highlighted the relevance of functional connectivity in further understanding the specificity of cognitive deficits in AD and how connectivity differentially influences the propagation of the different AD biomarkers. Finally, we conducted several studies on the links between lifestyle factors and neuroimaging biomarkers to unravel mechanisms of reserve. Further efforts are needed to better understand which lifestyle factor, or combination of factors, impact on AD pathology, and when, to help translating our knowledge to training programs that might prevent or delay brain and cognitive changes leading to AD dementia.

Keywords: Aging, Alzheimer's disease, diagnosis, disconnection, FDG-PET, lifestyle, meditation, multimodal neuroimaging, prevention, structural MRI

INTRODUCTION

Over the last twenty years, neuroimaging has increasingly contributed to major advances in Alzheimer's disease (AD), especially for the clinical diagnosis and to improve our understanding of the pathophysiological mechanisms of the disease.

The contribution of neuroimaging to advances in AD diagnosis is well illustrated by the fact that the three most established neuroimaging markers for AD (hippocampal atrophy, temporo-parietal hypometabolism, and cortical amyloid-β (Aβ) deposition) have been recently included in the revised criteria for AD [1–5]; their presence increases the

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likelihood of AD etiology, including in predementia stages, e.g., in mild cognitive impairment (MCI). Nevertheless, progress is still needed to understand the use of these biomarkers and how they should be combined in the different stages of the disease. Moreover, more refined biomarkers are needed to increase the specificity and the sensitivity of the diagnosis, especially in early stages.

The pathophysiological mechanisms of AD are not fully understood. The two main neuropathological landmarks of AD are Aβ and tau-neurofibrillary tangles pathologies. Their relative role and sequence is still debated. The main hypothesis is that Aβ accumulation is the (only) causative agent of AD pathology and that tau-neurofibrillary tangles, neuronal dysfunction, cell loss, vascular damage, cognitive deterioration, and dementia follow as a direct result of this initial Aβ deposition [6, 7]. This position is yet challenged by recent neuroimaging evidence with Aβ PET imaging (e.g., using PIB or Florbetapir) highlighting Aβ-independent tau-related neuronal injury [8–10]. Another key element in the study of AD mechanisms—and especially when considering the topography and propagation of the lesions—is to consider the structural and functional architecture of the normal brain. Indeed, neuroimaging studies have shown that neurodegenerative diseases initially target, and then spread within pre-existing brain networks (i.e., interconnected brain regions), which leads to a novel concept called the network degeneration hypothesis [11–13].

Over and above clinical diagnosis and mechanisms understanding, the development of new therapeutic strategies is urgently needed. As mentioned above, AD is a multifactorial disease that likely results from the complex interplay of multiple pathological processes, under the influence of internal and external determinants. The repeated failure of clinical trials strengthens the need to develop global strategies that may prevent, delay, and/or downregulate several of these AD pathological processes. In this context, there is a growing interest in the impact of modifiable environmental or lifestyle factors not only on AD but also more generally on cognition, mental health, and wellbeing in the aging population. Neuroimaging participates in these rising developments by providing tools to test the relationships between these factors and biomarkers of aging and AD and to monitor the effects of interventions based on lifestyle changes.

I have been asked to contribute an article focused on the implications of my work on AD, and where I see, or would like to see, the field moving in the future. The three following sections will thus give an overview of the contribution of the research I conducted with my team to the three main areas of investigation in AD research mentioned above, namely, early diagnosis of AD, elucidation of its pathophysiological mechanisms, and assessment of lifestyle factors for development of intervention strategies. These works and more generally recent advances in the field has led to new perspectives and questions that pave the way of future research. The last section will be dedicated to my perspective on the future of AD research and gives examples of ongoing and future research projects that we are running or would like to run in my laboratory.

EARLY DIAGNOSIS OF AD

Hippocampal subfield volumetry

Hippocampal atrophy is well-known as an early biomarker of AD, but it lacks specificity as it is also observed in many different situations, such as normal aging and several neurologic and psychiatric disorders including other neurodegenerative diseases (e.g., frontotemporal dementia, including semantic dementia (SD) [14]). Neuropathological studies have shown that hippocampal subfields (subiculum, CA1-4, and dentate gyrus) are differentially vulnerable to AD; hippocampal subfield volumetry may thus prove to be more accurate than global hippocampal volumetry to detect AD. This has been confirmed in an early work where we used a voxelwise analyses coupled with 3D hippocampal surface mapping to illustrate the discrepancies between the effects of AD versus normal aging on hippocampal subfield volumes, with a preferential involvement of the CA1 subfield versus the subiculum, respectively [15] (Fig. 1A. B). The same approach allowed us to illustrate the specificity of the relationships between hippocampal subfield volumes and episodic memory deficits in MCI patients [16]. These findings encouraged us to optimize a proton density sequence for very high resolution acquisition of the hippocampus and to develop guidelines for hippocampal subfield delineation [17] (Fig. 1C-E). Using this improved technology, we showed the differential involvement of the hippocampal subfields in normal aging, MCI, AD, and SD [17–19]. Thus, a linear effect of normal aging was observed on the subiculum from 20 to 90 years old, while the effect on CA1 volume was non-linear with a decrease starting from 50 years old only [19] (Fig. 1F). CA1 was the most sensitive subfield in early AD with higher

accuracy to discriminate between MCI and cognitively normal elderly than the whole hippocampus [18]. By comparison, SD was characterized by an hemispheric and antero–posterior asymmetry, significantly more marked than in AD, with greater involvement of the left and anterior hippocampal subfields [18]. Coupled with resting-state functional MRI, this approach also allowed us to highlight the specificities in hippocampal subfield intrinsic connectivity with the cerebral cortex in healthy elderly as well as their changes in patients with amnestic MCI [19].

Altogether, these studies and other works conducted worldwide on hippocampal subfields in AD highlight the relevance of high-resolution hippocampal acquisition in the early and differential diagnosis of AD. An international collaborative project has developed, the Hippocampal Subfields Group, aiming at standardizing hippocampal subfield delineation and promoting research on this field [20].

AD diagnosis and multimodal neuroimaging

Over and above hippocampal atrophy, other neuroimaging measures are known to be altered in AD. The most recognized ones, which have been integrated in the revised AD criteria [1–5], are hypometabolism in posterior cingulate and temporoparietal areas as measured with FDG-PET, and cortical Aβ deposition measured with PET and different Aβ-binding tracers such as florbetapir. According to the amyloid cascade hypothesis, Aβ deposition is supposed to appear first, then followed by atrophy and/or hypometabolism (both considered as markers of neurodegeneration) [5, 8]. We have, however, proposed an alternative perspective of the neuropathological processes of the disease which has implications for the use of the neuroimaging biomarkers of AD [21–23] (Fig. 2). In our perspective, all neuroimaging biomarkers should be

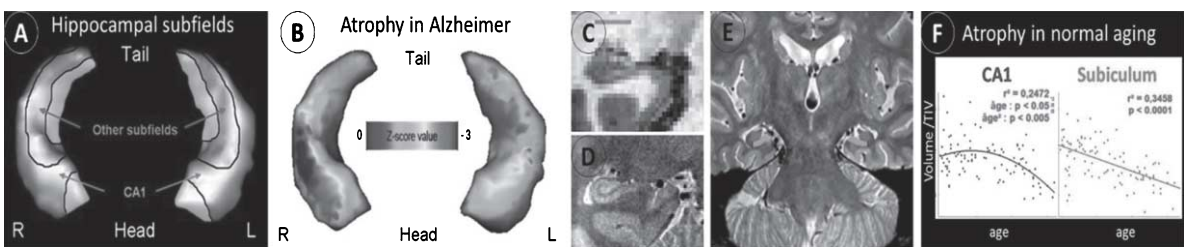

Fig. 1. Differential alteration of hippocampal subfields in AD versus normal aging. The hippocampal subfields can be distinguished on 3D hippocampal surface views (A), and this technique showed predominant atrophy of the CA1 subfield in AD (B). Compared to standard resolution T1 MRI (C), a high-resolution proton density MRI sequence allows to visualize the hippocampus fine anatomy (D) and thus to delineate the different hippocampal subfields (E). This approach is promising for early AD diagnosis as it allows to distinguish the effects of AD from that of other conditions such as normal aging (F).

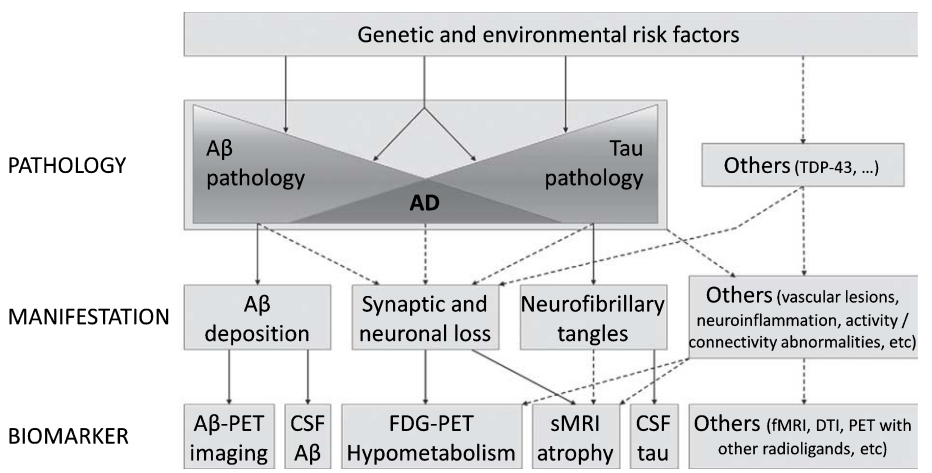

Fig. 2. Hypothetical model illustrating the links between the main AD biomarkers and the underlying neuropathological processes. In this multidetermined perspective of the disease, Aβ and tau pathologies appear as at least partly independent processes, under the influence of genetic and environmental factors, and interact to lead to AD disease. Other neuropathological processes, some of which are still unknown, are likely also involved in the physiopathology of the disease. Adapted from [21, 22].

considered to the same degree (rather than sequentially), their presence being associated with an incremental increase in the risk of AD pathophysiology and of progression to AD dementia. To test this hypothesis, we used neuropsychological, structural MRI, FDG-PET, and florbetapir-PET data in cognitively intact elderly individuals [24]. We showed that atrophy and hypometabolism biomarkers provide independent and complementary rather than redundant information, and that cognitively normal elderly tend to have either neurodegeneration or Aβ deposition but not both, suggesting additive rather than sequential/causative links between AD neuroimaging biomarkers. These works argue for the use of neuroimaging biomarkers as partly independent evidences increasing the likelihood of AD etiology.

Subjective cognitive decline (SCD)

The challenge in AD research is to diagnose the disease as early as possible to be able to assess the earliest pathophysiological processes and to intervene when the neurodegenerative process is still limited. The field has thus progressively moved towards the earliest stages such as subjective cognitive decline (SCD). Specific processes could be highlighted in this early stage; for instance, using data from the AIBL study in Melbourne, we found a specific relationship between atrophy and Aβ deposition in patients with SCD, but not in controls, MCI, or AD patients [25]. In an independent cohort of patients from the IMAP+ study in Caen, we showed that SCD was associated with hippocampal atrophy only when recruited from a memory clinic [26]. We also found that the same patients showed a profile of hippocampal subfield atrophy similar to that observed in AD and different from cognitively intact elderly (see above; [27]). Finally, we showed that detailed evaluation of SCD could provide accessible indication of the presence of cerebral Aβ or cognitive deficits [28]. Thus, we showed that specific SCD items (notably related with temporal disorientation) were associated with the presence of memory deficits in patients consulting at a memory clinic, and that stronger SCD (including for memory and attention) was associated with the presence of cortical Aβ deposition only in the asymptomatic elderly.

In sum, our research using neuroimaging biomarkers strengthens the view that SCD may represent a predementia stage of AD. We showed that the profile of brain atrophy associated with SCD resembles that observed in AD. However, all SCD patients will not develop AD and the challenge of future research will be to detect the pre-AD SCD. Developments in this field will be facilitated by the international initiative on SCD recently developed by expert researchers in the field [29].

Asymptomatic elderly carrying the APOE4 allele

The ε4 allele of the APOE (APOE4) is the major known genetic risk factor for late-onset AD [30]. Assessing brain changes in APOE4 carriers versus non-carriers in presymptomatic stages might help identifying early AD biomarkers. Based on a review of previous literature in the field, we proposed that APOE4 has a graded effect on the different AD biomarkers, with a predominant effect on Aβ deposition over brain structure and glucose metabolism [31] (Fig. 3). This view was supported by a study where we measured the effects of APOE, age, and the interaction between age and APOE on structural-MRI, FDG-PET, and Florbetapir-PET to provide a comprehensive and comparative assessment of APOE4 effects across the lifespan [32]. Thus, although decreases in brain volume and glucose metabolism with age tended to be stronger in noncarriers than in carriers, the difference between groups was not significant, while Aβ deposition was significantly higher, and increased faster with age, in carriers than non-carriers. These results reinforce the view that

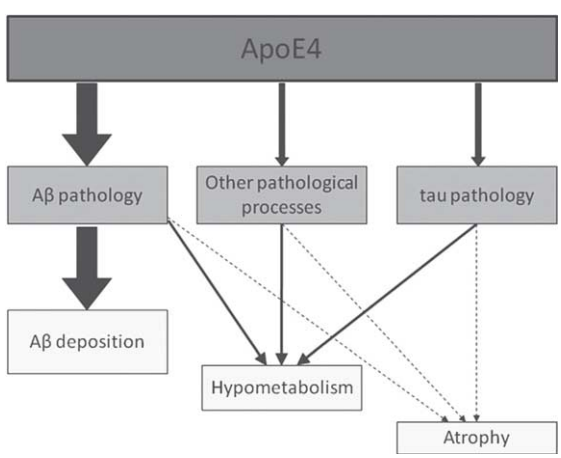

Fig. 3. Schematic representation of the graded effect of APOE4 on structural MRI (atrophy), FDG-PET (metabolism), and molecular (Aβ deposition) cortical changes. APOE4 effects clearly predominate on Aβ deposition (thick arrows), while the effects are more modest on cortical metabolism and volume (thin arrows). This figure also illustrates that APOE4 operates through both Aβ-dependent and Aβ-independent processes. From [31].

APOE4 mainly influences Aβ deposition, while the effects on neurodegeneration are at best subtle.

UNDERSTANDING AD PATHOPHYSIOLOGICAL MECHANISMS

Multimodal imaging provides a unique opportunity to investigate the temporal and topographical relationship between distinct pathological variables, and thus improve our understanding of pathophysiological interactions *in vivo*. Working with multimodal neuroimaging techniques for 15 years, we developed original analysis techniques to take full advantage of the complementarity of the different neuroimaging modalities.

Role of cortical Aβ deposition

Working on the AIBL data together with Victor Villemagne and Christopher Rowe in Melbourne, we conducted a series of studies aiming to further our understanding of the role of Aβ deposition in AD pathophysiology, throughout different stages of the disease and in relation with atrophy and cognitive deficits. We showed that Aβ deposition was only poorly related with local atrophy (i.e., only in SCD patients in the posterior cingulate cortex) [25]. We found a reverse relationship in cognitively intact elderly such that those with Aβ deposition tended to have greater temporal volume (which might reflect brain reserve, see also below) [33]. Moreover, we showed that hippocampal atrophy and neocortical Aβ deposition both independently predicted episodic memory performances in non-demented individuals [34]. The presence of Aβ deposition in the neocortex of cognitively normal elderly was also associated with increased rate of brain cortical atrophy within the next two years [35] (Fig. 4). Finally, we demonstrated that the rate of Aβ accumulation varied according to the initial amount of Aβ deposition (i.e., higher rate was found in Aβ-positive compared to negative individuals) but not according to the cognitive state [36]. This series of works demonstrate that Aβ deposition only poorly explains local atrophy, but is associated with increased rate of atrophy over time. In other words, the presence of Aβ deposition is associated with a worse prognosis but the relationship between Aβ deposition and neurodegeneration is complex and indirect.

Interesting, using data acquired in Caen in asymptomatic young to middle age adults, we were able to show that a physiological accumulation of Aβ starting from young adulthood and predominating in temporal lobes superimposed to the well-known medial frontal and parietal Aβ accumulation in late adulthood and AD [37].

Finally, in a collaborative work including data from Caen, Melbourne, Amsterdam, and San Francisco, we were able to study a series of 40 patients with a pre-scan clinical diagnosis of AD dementia but who had a negative Aβ PET scan [38]. We assessed their clinical and demographic features, patterns of brain atrophy and hypometabolism, and longitudinal clinical trajectories compared to a group of Aβ-positive AD and Aβ-negative controls. The main conclusions were that 1) the diagnosis was changed after the Aβ PET scan in almost all non-amnestic Aβ-negative AD cases and the individual profiles of atrophy and glucose metabolism helped to find an alternative diagnosis, which was most often confirmed by the clinical follow-up; 2) in the amnestic Aβ-negative AD cases, however, an alternative diagnosis could not be found in almost half of the cases as, although they had no Aβ, they mimic AD dementia in their clinical presentation and trajectory. These cases could thus not

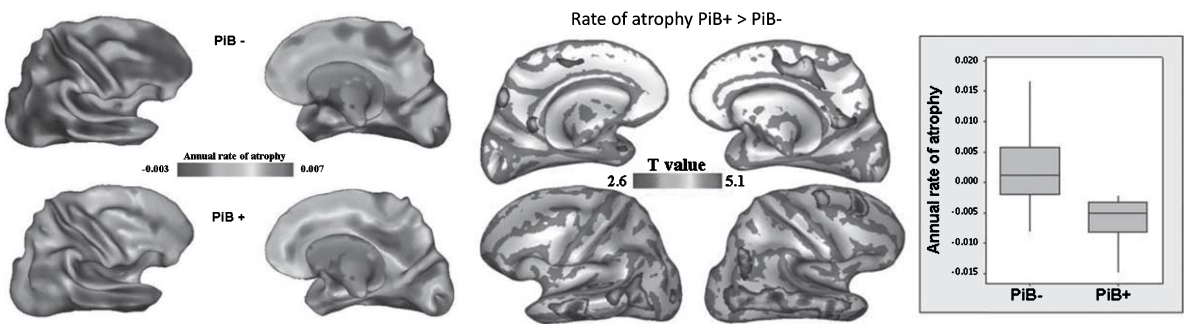

Fig. 4. Comparison of the rate of atrophy over two years between cognitively intact older adults with (PIB+) and without (PIB-) Aβ deposition in their brain (as measured with PIB-PET) (Left). This study shows a greater rate of atrophy in PIB+ individuals, especially in the temporal neocortex and posterior and middle cingulate cortex (Right). From [35].

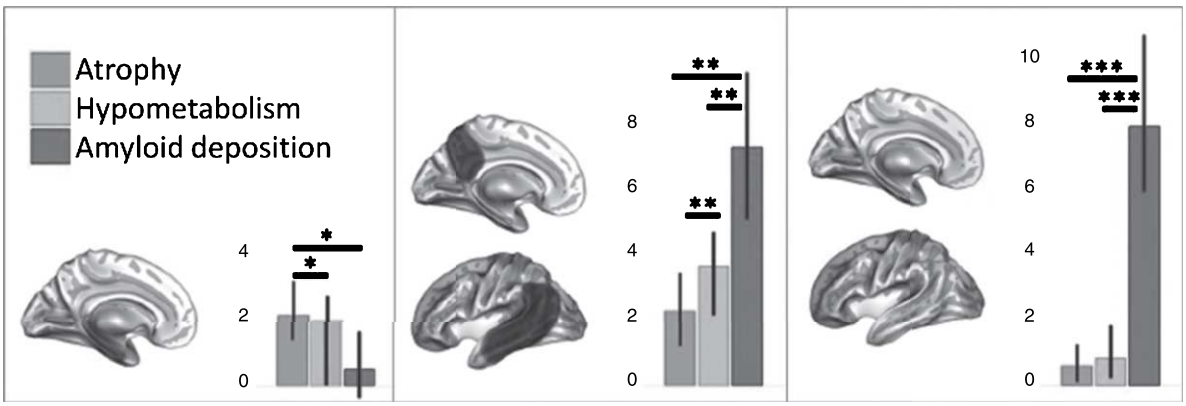

Fig. 5. Regional variation in the degree of biomarkers. Some regions show predominant atrophy (left panel), others have higher hypometabolism than atrophy (middle panel), and Aβ deposition predominates in other areas (right panel). This suggests differences in the underlying pathophysiological mechanisms. From [39].

be classified as AD based on the neuropathologic definition of this disease. This study emphasizes the need to define a clinical framework and terminology for the classification of these patients, who likely represent a mixed population of limbic-predominant AD-mimics.

Regional variations in the relative degree of the different AD biomarkers

In a series of works, we compared the relative degree of the different alterations using a method that we specifically developed for the purpose of multimodal neuroimaging analyses [39, 40]. This allowed us to highlight differences in the degree of atrophy, hypometabolism, and Aβ deposition across brain regions. We thus found that the hippocampus showed disproportionate atrophy (intermediate level of hypometabolism and almost no Aβ deposition), posterior associative temporal and parietal cortical areas showed disproportionate hypometabolism compared with atrophy (and important degree of Aβ deposition), while the frontal cortex was characterized by very high Aβ deposition and relatively weak atrophy and hypometabolism (Fig. 5). Interestingly, we showed in a more recent work that the expression of these patterns varied across different groups of patients at-risk for AD [41]. Thus, in SCD patients only the atrophy-predominant pattern was detected, while APOE4 carriers only demonstrated the frontal amyloid-predominant pattern. These findings altogether suggest that there might be different underlying mechanisms, and maybe different sequences, in the different groups of brain regions and across different at-risk populations.

Local and distant relationships between the different neuroimaging biomarkers

In a series of studies, we investigated the local and distant relationships between the different biomarkers. This allowed us to demonstrate that hypometabolism correlates with local atrophy (by contrast to Aβ deposition) suggesting that both alterations share at least partly common underlying mechanisms [40]. A significant proportion of hypometabolism and atrophy remains unrelated though [42]. We showed that disproportionate hypometabolism at least partly reflects diaschisis mechanisms, i.e., long distant effect of hippocampal atrophy on disconnected brain areas [43]. In a follow-up study, we used longitudinal neuroimaging data to provide support for the sequence of events and their causality [44] (Fig. 6).

The role of intrinsic connectivity in the pathophysiology of AD and SD

In addition to tau and Aβ pathologies, another key element in the pathophysiology of AD is brain structural and functional connectivity. Over and above diaschisis mechanisms mentioned above, other works conducted in our laboratory offered evidence for this purpose. First, we showed in cognitively intact individuals that functional connectivity measured with resting-state fMRI (within the default mode network that is especially relevant in AD) was related to cognitive performance, specifically in autobiographical memory, and not with inner experience [45]. We also showed the relevance of functional connectivity to explain certain cognitive manifestation of

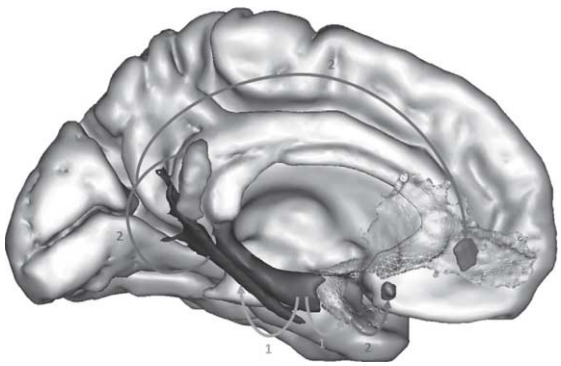

Fig. 6. Distant relationships between atrophy and hypometabolism in AD. Using original methods especially developed for this purpose, we showed that hippocampal atrophy (red) was at least partly responsible for the disruption of white matter fibers (the perforant path in blue and the uncinate fasciculus in yellow) (1) itself responsible for hypometabolism in the posterior cingulate (green) and medial orbitofrontal cortex (purple and light blue) (2). From [42].

AD such as anosognosia, i.e., the lack of consciousness of cognitive (memory) deficits [46]. Thus, we showed that anosognosia in AD results from a disruption of the communication between memory-related and the self-related brain networks. Using multiple imaging techniques including functional connectivity with resting-state fMRI, we also investigated the paradox of SD, i.e., the intriguing relative preservation of episodic memory in SD despite similar degree of hippocampal atrophy compared with AD [12]. We found that both diseases affect brain regions that are connected to the hippocampus, but only the connectivity with brain regions affected in AD are important for episodic memory in healthy individuals. We also showed that both diseases target different hippocampal networks, probably because they differentially affect the anterior versus posterior parts of the hippocampus, which are known to be connected to different brain regions [12]. This hypothesis found support in a recent evidence showing that the atrophy common to both AD and SD is associated with alterations in different white matter tracts, i.e., mainly the cingulum and corpus callosum in AD versus the uncinate and inferior longitudinal fasciculi in SD [47] (Fig. 7).

There is growing recognition for the relevance of connectivity in the propagation of the disease; thus, neuroimaging studies have shown that neurodegenerative diseases target brain networks (i.e., interconnected brain regions), which leads to a novel concept called the network degeneration hypothesis [11–13]. We also explored how much connectivity influences the topography and propagation of lesions in AD. We contributed to this area in showing that the influence of brain connectivity in AD lesion propagation depends on the neuroimaging modality. Thus, first using a cross sectional design, we showed that atrophy and intrinsic connectivity disruption were only present in the ventral posterior cingulate cortex (PCC) in MCI patients and spread to the dorsal PCC network in AD patients, while hypometabolism was present in both networks since the aMCI stage, possibly reflecting not only local disruption but also distant synaptic dysfunction [48]. Then we used longitudinal multimodal neuroimaging data in AD patients and showed that atrophy spread in regions with high specific connectivity, consistently with the transneuronal propagation hypothesis, while hypometabolism propagated in areas showing high global connectivity (that were also more vulnerable to Aβ deposition), in line with the hypothesis of higher vulnerability of hubs to hypometabolism and Aβ deposition [49].

LIFESTYLE VERSUS AD NEUROIMAGING BIOMARKERS

The alternative model we proposed on the pathophysiological mechanisms of AD [21, 22] (Fig. 2) recognizes the influence of environmental factors on AD pathophysiological mechanisms and processes. As a matter of fact, there is growing evidence in the literature that we could modify the course of the disease, and brain and mental health in general, by modifying our lifestyle [50–53]. We showed for example that higher education was able to counteract the effects of APOE ε4 on metabolism independently of Aβ deposition, as increased metabolism with education was found in APOE ε4 carriers in critical regions that sustain episodic memory performance [54]. In another study, we assessed the links between lifestyle factors and different neuroimaging measures including markers of AD. Thus, assessing the relationships between years of education and brain volume, metabolism, and connectivity, we showed that, in healthy elderly with no evidence for Aβ deposition, there was a positive relationship between education and brain volume and metabolism, especially in the anterior cingulate cortex [55]. Moreover, the connectivity of this region increased with increasing years of education especially with the hippocampus and posterior cingulate cortex, two regions particularly important in AD. By contrast, in a collaborative

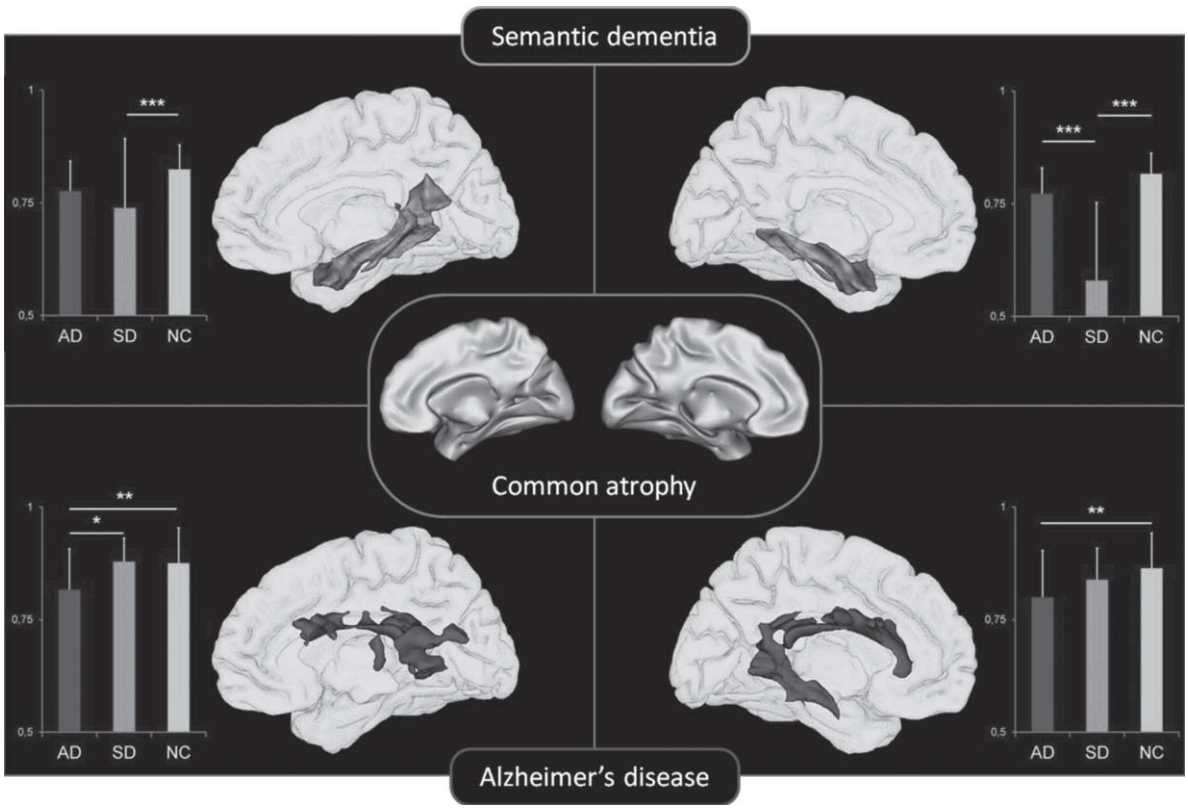

Fig. 7. Relationships between medial temporal lobe atrophy common to AD and SD (center panel) and whole-brain white matter density maps in patients with AD (top panel) and semantic dementia (bottom panel). From [45].

project, we found negative relationships between education and brain metabolism and connectivity in asymptomatic older adults including individuals with Aβ deposition [56]. We think that these apparently discrepant findings with positive versus negative relationships, also found in the literature, reflect the progression from neuroprotective to compensation processes over the course of the disease, which we summarized in an integrative model [57] (Fig. 8). Thus, in individuals without AD lesions, education is related with increased brain performances while when AD-related pathology appears, education is related with increased resistance to brain lesions so that at the same level of cognitive impairment, more lesions will be found in those with higher education. This model was supported by a recent study where we showed that higher education was associated with lower Aβ deposition in normal older adults but with higher Aβ deposition in MCI [58]. Moreover, in the same study we found increased FDG-PET uptake with education in MCI patients within the regions of higher Florbetapir-PET uptake, suggesting a compensatory increase in glucose metabolism. The findings suggest that early intellectual enrichment before the onset of dementia may be associated with protection in healthy asymptomatic elderly, and then with compensation from Aβ at the symptomatic stage.

Another relevant aspect to be further investigated in this area is the relative impact of different lifestyle factors. We started to assess this question by investigating the specific relationships between cognitive versus physical activity engagement during late-adulthood and gray matter volume in normal older adults. We showed independent relationships of the two lifestyle factors in both common and distinct brain areas, and found that the effects of late life cognitive and physical activity were independent from early cognitive engagement as reflected by years of education [59]. Further works are needed to understand the specific and synergic effects of different lifestyle factors, in different lifetime periods, as this information is crucial to design optimal non-pharmacological (preventive and therapeutic) intervention programs.

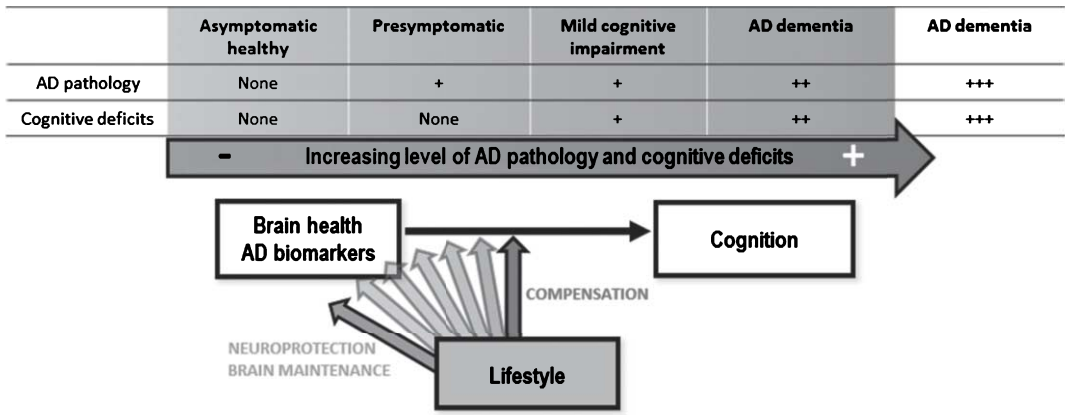

Fig. 8. Schematic theoretical representation of the differential expression of reserve mechanisms (neuroprotection versus compensation) across the spectrum from cognitively normal healthy adults to AD dementia. We propose that neuroprotection and brain maintenance predominates in healthy elderly while compensation processes predominate as AD progresses to dementia (probably up to a certain stage where compensation is not possible anymore). [54, 55].

FUTURE DIRECTIONS

There is more and more acknowledgment that AD is a multifactorial disease resulting from the contribution of, and interaction between, several pathological factors. Hence, we need to consider developing further specific markers of these pathologies; tau-PET imaging is particularly challenging but significant progress has been made [60]. Specific markers of other pathological processes including TDP-43, inflammation, α-synuclein, etc., are also awaited. This is important both to improve the diagnosis of neurodegenerative diseases, but also to understand the physiopathological processes leading to the disease. More specifically, applying the multimodal analysis methods described above to multiple neuroimaging techniques targeting specific pathological processes, especially within longitudinal design, would allow us to obtain a comprehensive picture of their relative role, sequence, and causal relationships. Further works on the role of brain connectivity in the propagation of the different pathological processes are needed to understand the relative contribution of several predictors. This offers relevant lines for future research as it might help us to develop strategies to slow down or even stop the propagation process.

In parallel, efforts should continue to develop biomarkers that are more widely available and less expensive than PET and that do not necessitate radioactivity exposure, especially for clinical application. For instance, previous works point toward the potential for MRI-based biomarkers including hippocampal subfield volumetry and MRI-based proxies of brain metabolism and Aβ deposition (e.g., perfusion MRI and susceptibility-weighted imaging) as promising biomarkers for AD. Research efforts are needed for the optimization, validation, and standardization of these approaches for clinical use. This is on-going for instance with the international Hippocampal Subfields Group (http://www.hippocampalsubfields.com).

For improvement of early diagnosis, the field has progressively moved from the MCI to the SCD stage. More studies are needed in this direction to better understand and differentiate the several possible causes for SCD based on neuroimaging but also on refined cognitive, self-assessment, and psychoaffective measures. These developments are crucial not only for early AD diagnosis, but also more generally to provide better care for those elderly who are more and more worried about getting AD. The international SCD Initiative would help promoting these developments [29].

The development of treatments is still a priority for future research to prevent, delay, slow down, or halt the degenerative process. Innovative strategies should be developed, new paths should be considered, and pharmacological therapeutics should take into account the multifactorial dimension of the disease instead of treating one of the element. Besides, as we recognize the impact of environmental, lifestyle, and psychoaffective factors on AD risk, we should seize the opportunity to translate our knowledge to training programs that might prevent or delay brain and cognitive changes leading to AD dementia. The growing interest for these approaches is reflected in

the recent advent of international initiatives, groups of experts, and meetings to promote research in this field (e.g., ISTAART professional interest area on Reserve, Resilience and Protective Factors and on non-pharmacological interventions: https://act.alz.org/site/SPageServer?pagename=ISTAART_PIA; 1st International Conference on Cognitive Reserve in the Dementias (ResDem): http://resdem2017.com/). Cognitive training programs, but also interventions based on physical or artistic activities, are being developed. Psychoaffective factors are less often considered, although stress, anxiety, and depression, all related to cognitive and sleep difficulties, are associated with increased risk for AD [61–63]. There is increased acknowledgment in the role for sleep in the physiopathology of the disease and we contributed to this knowledge showing specific relationships with neuroimaging markers [64], though more research is needed in this direction. Mental training for stress reduction and emotion regulation through meditation practice for instance might thus be particularly beneficial to elderly populations in reducing AD risk. In a pilot study, we showed that elderly expert meditators had higher gray matter volume and/or FDG metabolism compared to age-matched non-meditators in several frontal and parietal areas particularly sensitive to aging or AD effects [65]. These findings are encouraging as they suggest that meditation practice could reduce age-associated structural and functional brain changes. We are running a large European project including clinical trials assessing the effects of short and long-term meditation practice versus English learning and health education programs in elderly populations at-risk for AD (https://silversantestudy.fr/). Further works are needed to understand the specific and synergic effects of different lifestyle factors, in different lifetime periods, as this information is crucial to design optimal non-pharmacological (preventive and therapeutic) intervention programs.

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Preclinical Alzheimer's Disease: Implications for Refinement of the Concept

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Abstract. Increasing interest in clinical trials and clinical research settings to identify Alzheimer's disease (AD) in the earliest stages of the disease has led to the concept of preclinical AD. Individuals with preclinical AD have AD pathology without clinical symptoms yet. Accumulating evidence has shown that biomarkers can identify preclinical AD and that preclinical AD is associated with a poor clinical outcome. Little is known yet about the role of vascular and lifestyle risk factors in the development of preclinical AD. In order to better understand preclinical AD pathology and clinical progression rates, there is a need to refine the concept of preclinical AD. This will be of great value for advancements in future research, clinical trials, and eventually clinical practice.

Keywords: Amyloid, biomarkers, clinical trials, cognition, diagnosis, lifestyle, neuronal injury, preclinical Alzheimer's disease, prognosis, vascular risk

INTRODUCTION

Over the last two decades the developments in biomarkers research have completely altered our perception of Alzheimer's disease (AD). It was shown that amyloid-β (Aβ) abnormalities can be observed more than 15 years before clinical symptoms [1–3]. This led to the introduction of the diagnostic category of preclinical AD, defined as individuals with abnormal Aβ but normal cognition. The concept of preclinical AD opens a wide range of possibilities for research of the development of AD and ultimately the prevention of dementia. In this paper, we give an overview of the diagnostic criteria of preclinical AD and the prevalence, clinical outcome, and risk factors of preclinical AD. We conclude with a discussion of the impact of the concept of preclinical AD on future research, trial design, and clinical practice.

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DIAGNOSIS OF PRECLINICAL AD

AD is characterized in the brain by extracellular plaques, resulting from aggregation of the Aβ protein, followed by intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein, and brain atrophy. In 2011, a working group of the National Institute on Aging and Alzheimer's Association (NIA-AA) proposed three ordered biomarker-based stages for preclinical AD for cognitively normal individuals with abnormal Aβ [4]. Stage 1 preclinical AD was defined as the presence of only an abnormal Aβ marker, stage 2 as abnormal Aβ and at least one abnormal neuronal injury marker, and stage 3 as

abnormal Aβ, neuronal injury, and subtle cognitive changes without impairments.

Aβ biomarkers

Aβ aggregation can be measured in cerebrospinal fluid (CSF) or on positron emission tomography (PET). Individuals with AD have lower $A\beta_{42}$ levels or a lower ratio of $A\beta_{42}$ to $A\beta_{40}$ in CSF and an increased PET Aβ tracer binding [5, 6]. CSF and PET Aβ measures are not interchangeable as several studies reported discordance in abnormality between both measures in cognitively normal individuals (discordance range 8–21% [7–10], with typically individuals having abnormal CSF $A\beta_{42}$ but normal Aβ PET. This suggests that CSF $A\beta_{42}$ may identify the disease before Aβ PET binding becomes abnormal. Some studies have demonstrated that the ratio of CSF $A\beta_{42}/A\beta_{40}$ may show a better concordance with Aβ PET, compared to CSF $A\beta_{42}$ alone, in particular for specific assays [11, 12].

Neuronal injury markers

Tau pathophysiology can be measured in CSF by p-tau levels and recently also on PET by tau PET tracers. Other AD-related neuronal injury markers include medial temporal lobe (MTL) atrophy on magnetic resonance imaging (MRI), hypometabolism on fluodeoxyglucose (FDG) PET, and higher levels of total tau (t-tau) in CSF. Whereas p-tau in CSF and tau on PET are thought to be specific markers of AD pathophysiology, MTL atrophy, hypometabolism on FDG-PET, and higher levels of t-tau are also seen in other conditions and therefore considered nonspecific to AD. Also neuronal injury markers are not interchangeable as they measure different processes [9, 13]. This is reflected in the higher discordance in abnormality between these markers. We reported 41% discordance for CSF t-tau and hippocampal atrophy [9], while another study found 15% discordance for CSF t-tau and FDG-PET and 26% for FDG-PET and hippocampal atrophy [10]. When AD-related atrophy patterns were studied, a discordance of 21% with CSF t-tau and 49% with FDG-PET was found, whereas CSF t-tau and p-tau showed a discordance of 49% with FDG-PET [14].

PREVALENCE OF PRECLINICAL AD

Around one-third of individuals in the general elderly population have Aβ pathology. A recent worldwide meta-analysis based on over 50 studies showed that Aβ prevalence increased from age 50 to age 90 from 10% to 44% [3].

The prevalence of preclinical AD NIA-AA criteria stage 1 ranged from 8 to 21%, and of stage 2 from 8 to 34% (Table 1) [2, 14–21]. Differences in prevalence between studies are most likely related to differences in setting, age, kind of biomarkers (e.g., imaging versus CSF markers), and cut-offs that were used. Some studies specifically defined subtle cognitive decline for stage 3 and found a prevalence of 2 to 4% for this stage (Table 1). However, there is no clear consensus yet on defining subtle cognitive change at the preclinical AD stage. In our head-to-head comparison study, using CSF markers for classification resulted in a prevalence of preclinical AD stage 1 of 12%, and stage 2 + 3 of 9%, while with imaging markers the prevalence of stage 1 was 20% and stage 2 + 3 8% [9]. Of the individuals in stage 1 according to CSF biomarkers, 19% were in stage 2 + 3, and 39% were normal according to imaging biomarkers, whereas of the individuals in stage 2 according to CSF biomarkers, 74% were in stage 1, and 11% were normal according to imaging biomarkers.

OUTCOME OF PRECLINICAL AD

Studies examining the relation between Aβ pathophysiology and longitudinal clinical outcome remain scarce. Overall, Aβ pathophysiology in cognitively normal individuals has been associated with an increased progression rate to mild cognitive impairment (MCI) and AD dementia [1, 2, 22–24]. Some studies have investigated the association between Aβ pathophysiology and decline in specific cognitive domains. These findings were examined in a recent meta-analysis (overall $n = 14$ studies) and show that Aβ pathophysiology is associated with a small to moderate decline in global cognition (Cohen's $d = 0.30$), with smaller effects for semantic memory, visuospatial function, and episodic memory (Cohen's $d = 0.24$), while no such association was found with working memory, processing speed, and executive function [25]. Given the relatively small effects, more sensitive cognitive measures may be needed to capture early cognitive change. The preclinical Alzheimer cognitive composite (PACC) and Alzheimer's prevention initiative composite cognitive test score (APCC) have been suggested as sensitive tests for global cognitive decline early on in preclinical AD [24, 26, 27] and have been included

Table 1
Prevalence of preclinical AD by NIA-AA stage

Study	N	Setting	Age	Females	*APOE* ε4	Amyloid marker	Injury marker	Stage 0	Stage 1	Stage 2	Stage 3	Reference
ADC	132	Memory clinic	61.4 y (SD 8.3)	42%	41%	CSF $A\beta_{42}$	CSF t-tau or p-tau	61%	8%	8%		Van Harten et al., 2013 [16]
ADNI	326	Clinical trial sites	74.2 y (range 69–85)	47%	27%	CSF $A\beta_{42}$	CSF t-tau, HCV	32%	15%	22%	3%	Toledo et al., 2014 [14]
AIBL	573	Community dwelling	73.1 y (SD 6.2)	58%	49%	PiB PET	HCV	54%	15%	9%		Burnham et al., 2016 [18]
BIOCARD	222	Community dwelling via clinical setting	56.9 y (SD 10.1)	60%	33%	CSF $A\beta_{42}$	CSF t-tau or p-tau	46%	21%	13%		Soldan et al., 2016 [19]
GEM	140	Clinical trial setting	∼86.0 y (SD 2.9)	∼41%	∼19%	PiB PET	HCV	27%	19%	34%		Zhao et al., 2018 [21]
HABS	166	Community dwelling	74 y (IQR 68–79)	55%	30%	PiB PET	FDG PET or HCV	49%	11%	17%		Mormino et al., 2014 [17]
MCSA	296	Population-based	78 y (IQR 75–82)	44%	25%	PiB PET	FDG PET or HCV	43%	15%	13%	2%	Knopman et al., 2012 [15]
WU-ADRC	311	Community dwelling	72.9 y (SD 6.0)	55%	34%	CSF $A\beta_{42}$	CSF t-tau or p-tau	41%	15%	12%	4%	Vos et al., 2013 [2]

Aβ, amyloid-β; AD, Alzheimer's disease; ADC, Amsterdam Dementia Cohort; ADNI, Alzheimer's Disease Neuroimaging Initiative; AIBL, Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing; *APOE*, apolipoprotein E; BIOCARD, Biomarkers of Cognitive Decline Among Normal Individuals Study; CSF, cerebrospinal fluid; FDG, fluorodeoxyglucose; GEM, Ginkgo Evaluation of Memory Study; HABS, Harvard Aging Brain Study; HCV, hippocampal volume; MCSA, Mayo Clinic Study of Aging; PET, positron emission tomography; PiB, Pittsburgh compound B; P-tau, phosphorylated tau; T-tau, total tau; WU-ADRC, Washington University Knight Alzheimer's Disease Research Center.

Table 2
Preclinical AD NIA-AA stages and clinical outcome

Study	N	Setting	Age	Females	*APOE* ε4	Amyloid marker	Injury marker	Outcome measure	Follow-up time	Overall APR progression	Progression Stage 0	Progression Stage 1	Progression Stage 2	Progression Stage 3	Reference
ADC	132	Memory clinic	61.4 y (SD 8.3)	42%	41%	CSF $A\beta_{42}$	CSF t-tau or p-tau	MCI or AD dementia	1.8 y (1.3 SD)	10% overall	3%	18%	60%		Van Harten et al., 2013 [16]
										5.6% overall APR	*1.7% APR*	*10.0% APR*	*33.3% APR*		
ADNI	326	Clinical trial sites	74.2 y (range 69–85)	47%	27%	CSF $A\beta_{42}$	CSF t-tau, HCV	MCI or AD dementia	6 y (IQR 3.0–7.0)	13% overall (6.3% after 3 y and 17.0% after 5 y)	9% Ref	14% HR = 2.6	12% HR = 1.8	25% HR = 11.3	Toledo et al., 2014 [14]
										2.2% overall APR	*1.5% APR*	*2.3% APR*	*2.0% APR*	*4.2% APR*	
AIBL	573	Community dwelling	73.1 y (SD 6.2)	58%	49%	PiB PET	HCV	MCI or AD dementia	6 y	11% overall	8% Ref	16% HR = 2.27	24% HR = 5.60		Burnham et al., 2016 [18]
										1.8% overall APR	*1.3% APR*	*2.7% APR*	*4.0% APR*		
BIOCARD	222	Community dwelling via clinical setting	56.9 y (SD 10.1)	60%	33%	CSF $A\beta_{42}$	CSF t-tau or p-tau	Cognitive decline; MCI or AD dementia	11 y (0–18; SD 4.1)	23% overall	Similar to stage 1; Ttau 20% Ptau 18%	Similar to normal group; Ttau 20% Ptau 20%	Faster decline than other groups; Ttau 53% Ptau 53%		Soldan et al., 2016 [19]
										2.1% overall APR	*Ttau 1.8% Ptau 1.6% APR*	*Ttau 1.8% Ptau 1.8% APR*	*Ttau 4.8% Ptau 4.8% APR*		
GEM	140	Clinical trial setting	~86.0 y (SD 2.9)	~41%	~19%	PiB PET	HCV	Cognitive decline	12.2 y (SD 2.2; range 7.2–15.1)	–	Ref.	Faster decline	Fastest decline		Zhao et al., 2018 [21]
HABS	166	Community dwelling	74 y (IQR 68–79)	55%	30%	PiB PET	FDG PET or HCV	Cognitive decline	2.09 y (IQR 1.9–2.3)	–	No decline	No decline	Faster decline than other groups		Mormino et al., 2014 [17]
MCSA	296	Population-based	78 y (IQR 75–82)	44%	25%	PiB PET	FDG PET or HCV	MCI or dementia	1.3 y (range 1.1–5.1)	10% overall	5%	11%	21%	43%	Knopman et al., 2012 [15]
										7.7% overall APR	*3.8% APR*	*8.5% APR*	*16.2% APR*	*33.1% APR*	
WU-ADRC	311	Community dwelling	72.9 (SD 6.0)	55%	34%	CSF $A\beta_{42}$	CSF t-tau or p-tau	CDR ≥ 0.5 DAT	3.9 y (range 1–15)	10% overall	2% after 5 y Ref	11% after 5 y HR = 4.6	26% after 5 y HR = 14.3	56% after 5 y HR = 33.8	Vos et al., 2013 [2]
										2.6% overall APR	*0.4% APR*	*2.2% APR*	*5.2% APR*	*11.2% APR*	

Aβ, amyloid-β; AD, Alzheimer's disease; ADC, Amsterdam Dementia Cohort; ADNI, Alzheimer's Disease Neuroimaging Initiative; AIBL, Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing; *APOE*, apolipoprotein E; APR, annual progression rate; BIOCARD, Biomarkers of Cognitive Decline Among Normal Individuals Study; CDR, Clinical Dementia Rating; CSF, cerebrospinal fluid; DAT, Alzheimer-type dementia; FDG, fluorodeoxyglucose; GEM, Ginkgo Evaluation of Memory Study; HABS, Harvard Aging Brain Study; HCV, hippocampal volume; HR, hazard ratio; MCI, mild cognitive impairment; MCSA, Mayo Clinic Study of Aging; PET, positron emission tomography; PiB, Pittsburgh compound B; P-tau, phosphorylated tau; T-tau, total tau; WU-ADRC, Washington University Knight Alzheimer's Disease Research Center.

Table 3
Vascular and lifestyle risk factors for preclinical AD

Study	N	Setting	Age	Females	APOE ε4	Risk factor	Amyloid outcome measure	FU time	Predictive accuracy	Reference
Cross-sectional										
ADNI	112	Clinical trial settings	75.7 y (SD 5.2)	50%	23%	(Change in) BMI	CSF $A\beta_{42}$, t-tau and PiB PET	–	Lower BMI levels were associated with more CSF Aβ and tau burden. No association was found with BMI change	Vidoni et al., 2011 [28]
AIBL	116	Community dwelling	70.3 y (range 60–95)	54%	47%	Physical activity	PiB PET	–	Higher exercising ε4 carriers had less Aβ burden	Brown et al., 2013 [40]
AIBL	162	Community dwelling	∼69.8 y (SD 6.8)	∼59%	∼26%	Dietary protein and fiber intake	PiB PET	–	Higher protein intake was associated with less Aβ burden	Fernando et al., 2018 [37]
BAC	92	Community dwelling	75.2 y (SD 5.6)	63%	–	Lifetime cognitive activity, current physical activity	PiB PET, combined cortical thickness, FDG-PET and HCV score	–	Higher lifetime cognitive activity was associated with less Aβ burden	Wirth et al., 2014 [35]
Bonn cohort	87	Memory clinic	67.7 y (SD 9.1)	49%	23%	CSF cholesterol, cholesterol precursors, and cholesterol elimination products	CSF $A\beta_{42}$, p-tau	–	Cholesterol elimination products were only associated with p-tau levels	Popp et al., 2013 [32]
DESCRIPA	111	Memory-clinic setting	67.0 y (SD 7.6)	51%	46%	Social activity, physical activity, cognitive activity, alcohol consumption, current smoking, sleep problems	CSF $A\beta_{42}$, t-tau, p-tau and HCV	–	No effect was found	Reijs et al., 2017 [46]
DIAN	139 presymptomatic mutation carriers	Clinical trial settings	34.9 y	58%	28%	Leisure time exercise activity	CSF $A\beta_{42}$, t-tau PiB PET	–	Only in amyloid-positive individuals, higher exercise was associated with less Aβ on PET. A stronger association was found between Aβ PET and estimated years of onset in those with lower exercise	Brown et al., 2017 [43]
DIAN	120 presymptomatic mutation carriers	Clinical trial settings	35.3 y (SD 8.0)	73%	–	BMI	PiB PET	–	Lower BMI was associated with less years before estimated symptom onset and more Aβ burden	Müller et al., 2017 [29]
DLBS	118	Community dwelling	69.5 y (range 47–89 y)	–	23%	Hypertension	Florbetapir PET	–	Hypertension with 1 ε4 allele was associated with more Aβ burden	Rodrigue et al., 2013 [30]

(*Continued*)

Table 3
continued

Study	N	Setting	Age	Females	APOE ε4	Risk factor	Amyloid outcome measure	FU time	Predictive accuracy	Reference
FINGER	48	Population-based	52.4 y	∼47%	∼29%	Blood pressure, BMI, total and LDL cholesterol, and glucose homeostasis	PiB PET (42% abnormal)	–	No effect was found	Kemppainen et al., 2018 [34]
HABS	79	Community dwelling				Social isolation/ loneliness	PiB PET	–	Social isolation was associated with more Aβ burden	Donovan et al., 2016 [44]
HABS	186	Community dwelling	74 y (SD 6)	55%	31%	Recent and past cognitive activity, Recent physical activity, Objective recent walking activity	PiB PET, FDG-PET, HCV	–	No effect was found	Gidicsin et al., 2015 [45]
MCSA	430 (of which 38 were cognitively impaired)	Population-based	74.7 y (SD 8.4, range 60–98)	44%	28%	Hypertension, hyperlipidemia, cardiac-arrhythmias, coronary artery disease, congestive heart failure, diabetes mellitus, and stroke	PiB PET, FDG-PET, ERC tau-PET, AD atrophy patterns on MRI	–	Vascular health had direct and indirect impact on neurodegeneration but not Aβ, hyperlipidemia had a direct impact on tau	Vemuri et al., 2017 [33]
NYU-ADC	45	Community dwelling	54 y (SD 11)	71%	42%	Physical activity, Mediterranean diet	PiB PET, FDG-PET, atrophy on MRI	–	Higher physical activity and Mediterranean diet were associated with less AD pathology (Aβ/FDG/MRI). Combined higher physical activity and Mediterranean diet was associated with the least AD pathology	Matthews et al., 2014 [38]
NYU-ADC	52	Community dwelling	54 y (SD 11)	71%	47%	Nutrient patterns	PiB PET, FDG-PET, atrophy on MRI	–	Vitamin B12, vitamin D and zinc were associated with less AD pathology (Aβ/FDG/MRI). Such associations were also found with vitamin E and PUFA (FDG/MRI), anti-oxidants and fibers (FDG); Fats were associated with more abnormal FDG and MRI	Berti et al., 2015 [36]
UCLA	24	Community dwelling SCI	63.1 y (SD 11.6)	67%	33%	Physical activity, BMI, diet	Aβ /tau FDDNP-PET	–	Healthier diet was associated with less Aβ/tau binding	Merrill et al., 2016 [42]
UCSD/ UW/ OHSU	177	Community dwelling	69.4 y (SD 8.3, range 55–100)	58%	34%	Pulse pressure (systolic-diastolic blood pressure)	CSF $A\beta_{42}$ and p-tau	–	Elevated pulse pressure was associated with abnormal p-tau/$A\beta_{42}$ and p-tau	Nation et al., 2013 [31]

(*Continued*)

Table 3
continued

Study	N	Setting	Age	Females	APOE ε4	Risk factor	Amyloid outcome measure	FU time	Predictive accuracy	Reference
WRAP	186	Population-based	~61 y (SD 6)	~67%	~40%	Current physical activity	PiB PET, FDG-PET, HCV	–	With advancing age, physically active individuals had less AD pathology (Aβ/FDG/HCV) compared to the physically inactive	Okonkwo et al., 2014 [41]
WU-ADRC	165	Community dwelling	65.4 y	68%	34%	Physical exercise	CSF $A\beta_{42}$ + PiB PET	–	Lower physical activity was associated with more abnormal Aβ in CSF and on PET; on PET only in *APOE* ε4 carriers	Head et al., 2012 [39]
Semi-longitudinal (AD biomarkers only assessed at follow-up)										
ARIC	346	Community dwelling	52 y (range 45–64)	58%	31%	Midlife obesity, current smoking, hypertension, diabetes, and total cholesterol	Florbetapir PET (51% abnormal)	Median 23.5 y (IQR 23.0–24.3)	Only midlife obesity predicted Aβ as single factor (OR = 2.06) 0 factors Ref; 31% abnormal amyloid 1 factor OR = 1.88 (NS); 50% abnormal Aβ >= 2 factors OR = 2.88; 61% abnormal Aβ	Gottesman et al., 2017 [47]
BIOFINDER	318	Population-based	54 y (SD 4.7)	60%	28%	Midlife triglycerides, cholesterol, HDL, and LDL	CSF $A\beta_{42}$ and p-tau+ in subset flutemetamol PET ($n = 134$)	20 y (mean age individuals 73 y)	Higher triglycerides levels were associated with abnormal CSF $A\beta_{42}$ (OR = 1.34) and $A\beta_{42}$/p-tau (OR = 1.46) higher levels of LDL with abnormal Aβ PET (OR = 2.03–2.12), and higher levels of HDL with less abnormal Aβ PET (OR = 0.25)	Nagga et al., 2018 [49]
FINGER	48	Population-based	52.4 y	~47%	~29%	CAIDE dementia risk score: age, sex, years of formal education, systolic blood pressure, BMI, serum total cholesterol, and physical activity	PiB PET, HCV and MTA on MRI	17.6 y	The CAIDE risk score was only associated with more atrophy (HCV/MTA) at follow-up	Stephen et al., 2017 [50]

(*Continued*)

Table 3
continued

Study	N	Setting	Age	Females	APOE ε4	Risk factor	Amyloid outcome measure	FU time	Predictive accuracy	Reference
MCSA	942	Population-based	History age 40–64 y	45%	29%	Intellectual enrichment, midlife physical inactivity, obesity, ever smoked, diabetes, hypertension, and dyslipidemia, and late life cardiovascular and metabolic conditions	PiB-PET, AD MRI atrophy patterns	Current age 79.7 y (SD 5.9)	Only midlife dyslipidemia was associated with late life Aβ pathology. Obesity, smoking, diabetes, hypertension, and cardiac and metabolic conditions were associated with greater AD-pattern neurodegeneration	Vemuri et al., 2017 [48]
Longitudinal										
ADNI	229	Clinical trial sites	75.1 y (SD 5.0)	48%	27%	Framingham Heart Study risk score: including age, gender, body mass index, blood pressure, smoking, and diabetes	CSF $A\beta_{42}$, FDG-PET, HCV	3.2 y (SD 1.0)	Vascular burden was not associated with cross-sectional and longitudinal changes in Aβ, FDG, or HCV	Lo et al., 2012 [52]
MCSA	393 (of which 53 were cognitively impaired)	Population-based	78.6 y (SD 5.0)	38%	28%	Education, occupation, and reported midlife cognitive activity, exercise activity, and physical activity	PiB PET, FDG-PET, HCV	2.5 y (SD 1.2)	Among highly educated individuals, high midlife cognitive activity was associated with lower (longitudinal) Aβ burden in APOE ε4 carriers	Vemuri et al., 2016 [53]
NYU-ADC	77	Community dwelling	63.4 y (SD 9.4, range 44–86)	60%	30%	Mean arterial pressure in people with (32%) and without hypertension	CSF $A\beta_{42}$, t-tau, p-tau	2.0 y (SD 0.5)	Decreased mean arterial pressure was only related to longitudinal increase in p-tau in people with hypertension	Glodzik et al., 2014 [51]

Aβ, amyloid-β; ADNI, Alzheimer's Disease Neuroimaging Initiative; AIBL, Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing; *APOE*, apolipoprotein E; ARIC, Atherosclerosis Risk in Communities Study; BAC, Berkeley Aging Cohort; BIOCARD, Biomarkers of Cognitive Decline Among Normal Individuals Study; BIOFINDER, Biomarkers For Identifying Neurodegenerative Disorders Early and Reliably; BMI, body mass index; CSF, cerebrospinal fluid; DIAN, Dominantly Inherited Alzheimer Network; DESCRIPA, Development of screening guidelines and criteria for predementia Alzheimer's disease; DLBS, Dallas Lifespan Brain Study; ERC, entorhinal cortex; FDG, fluorodeoxyglucose; FINGER = Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability; HABS, Harvard Aging Brain Study; HCV, hippocampal volume; MCSA, Mayo Clinic Study of Aging; MRI, magnetic resonance imaging; MTA, medial temporal lope atrophy; NYU-ADC, New York University Alzheimer's Disease Center; PET, positron emission tomography; PiB, Pittsburgh compound B; P-tau, phosphorylated tau; T-tau, total tau; UCLA, University of California Los Angeles; UCSD/WU/OHSU, collaboration between University of California San Diego, Washington University, and Oregon Health & Science University; WRAP, Wisconsin Registry for Alzheimer's Prevention; WU-ADRC, Washington University Knight Alzheimer's Disease Research Center.

as primary outcome measure in the first clinical trials in preclinical AD. Future research on Aβ pathophysiology in relation to decline on the APCC and PACC and other cognitive tests will help to better understand the prognosis and outcome of Aβ pathophysiology in cognitively normal individuals.

Progression rates to cognitive impairment or dementia have been found to increase with advancing NIA-AA preclinical AD stage (Table 2) [2, 14–16, 18, 19]. Reported progression rates to cognitive impairment or dementia were 11–20% in stage 1, 12–26% in stage 2, and 25–56% in stage 3 after an average follow-up of 1 to 11 years (Table 2). Studies that combined stage 2 and stage 3 reported progression rates of 24–60%. A similar trend was found for decline in performance on cognitive testing with more decline in more advanced NIA-AA stages [17, 19, 21]. Moreover, our head-to-head comparison study of AD defined by CSF versus imaging markers (CSF $A\beta_{42}$ and tau versus amyloid PET and hippocampal atrophy) showed similar progression rates for preclinical AD defined by CSF and imaging markers [9], indicating that both modalities have comparable prognostic value. Together, these findings show that information on neuronal injury can help to further refine the prognosis of cognitively normal individuals with Aβ pathophysiology.

VASCULAR AND LIFESTYLE RISK FACTORS FOR PRECLINICAL AD

Vascular and lifestyle factors are established risk factors for AD-type dementia but little is known about the role of these factors in the development of AD pathology in cognitively normal individuals. Most previous studies were cross-sectional and findings have been conflicting. Lower BMI [28, 29], hypertension [30] and increased pulse pressure [31] were associated with Aβ pathology but other studies did not find an association between vascular risk factors and Aβ pathology [32–34] (Table 3).

Higher cognitive activity [35], a healthy nutrient pattern [36–38], higher physical activity [38–43], and higher social activity [44] were associated with less Aβ pathology in cognitively normal individuals. However, several studies did not find an association between these lifestyle activities and Aβ accumulation, e.g., [45, 46].

There are only few longitudinal studies available to date that have examined the relation between risk factors and development of amyloid pathology (Table 3). Most of these studies lacked a baseline measurement of Aβ pathology, which limits the interpretation of timing of events. The studies suggested that especially risk factors in midlife (40–65 years) were associated with AD pathology in late life. The ARIC study showed that having 2 or more of the midlife risk factors obesity, smoking, cholesterol, hypertension, and diabetes was associated with a higher prevalence of Aβ pathology in later life compared to having none of these midlife risk factors (∼60 versus 30%) [47]. Another population-based study suggested that midlife dyslipidemia was associated with Aβ pathology in later life while midlife obesity, smoking, diabetes, and hypertension were associated with AD-related neurodegeneration on imaging [48]. Midlife lipid levels were also found to be associated with late life Aβ pathophysiology in the BIOFINDER study [49]. The FINGER study reported that a higher midlife CAIDE risk profile (consisting of age, sex, education, blood pressure, cholesterol, body mass index, and physical inactivity) was associated with more pronounced vascular pathology and neurodegeneration on imaging in later life, while no association with Aβ accumulation was found, although this could be due to the relative small sample size (N = 48) [50]. A study with longitudinal biomarker assessment in individuals in midlife and later life found an association between decreased mean arterial pressure and an increase in p-tau over time but no association with Aβ changes [51], whereas another study did not find any relation between vascular burden and longitudinal biomarker changes [52]. Furthermore, among highly educated individuals, high midlife cognitive activity was found to be associated with less increased longitudinal Aβ on PET in APOE ε4 carriers [53]. Although the above findings are inconsistent, lack long-term longitudinal amyloid biomarker measurements, and are based on relatively small samples, they suggest that lifestyle and vascular risk factors may be involved in the development of AD-related pathology later in life.

ONGOING PRECLINICAL AD TRIALS

AD disease-modifying treatment targets are explored in a number of ongoing secondary prevention trials. Secondary prevention trials are testing disease-modifying drugs in individuals with preclinical AD, i.e., individuals with AD pathology but no clinical symptoms yet or presymptomatic mutation carriers. BACE inhibitors are the most commonly

used AD therapy agent and have shown robust reduction in Aβ pathology. Also immunotherapies, especially monoclonal antibodies, are used in several AD trials [54]. Ongoing trials in preclinical AD include, for example, the Anti-Amyloid treatment for Asymptomatic AD (A4) [55], EARLY, Alzheimer's Prevention Initiative (API) [56], and Dominantly Inherited Alzheimer's Network (DIAN) [57, 58]. The A4 trial and EARLY trial recruit cognitively normal individuals with evidence of Aβ pathology to test an anti-Aβ antibody and BACE inhibitor, respectively. The API recruits cognitively normal individuals at high risk of developing symptoms based on their genetic background. The API autosomal-dominant AD trial tests an anti-Aβ antibody in individuals with presenilin 1 (*PSEN1*) mutations close to their estimated age of onset, while the API *APOE* ε4 trial tests an Aβ vaccine or BACE inhibitor in cognitively normal homozygous *APOE* ε4 carriers. The DIAN trial tests an anti-Aβ antibody in autosomal dominant mutation carriers in *APP*, *PSEN1* and *PSEN2* genes. All these ongoing trials use a cognitive composite score as primary outcome measure.

IMPACT OF PRECLINICAL AD ON FUTURE RESEARCH, TRIALS, AND CLINICAL PRACTICE

The concept of preclinical AD has had and will continue to have an enormous impact on developments in research, trials, and clinical practice. Still, the concept of preclinical AD needs to be further refined and several challenges that come with the concept of preclinical AD remain to be tackled.

Need of refinement of preclinical AD

Refinement of the preclinical AD concept is needed in order to capture the earliest changes of AD and understand how the disease unfolds. The proposed ATN classification system forms a first step toward refining preclinical AD by differentiating between several neuronal injury markers [59]. Here the A stands for Aβ pathology (CSF or PET), the T for tau pathology (CSF p-tau, tau PET), and the N for other forms of neuronal injury (hippocampal volume, CSF t-tau, FDG-PET). This classification system is implemented in the new proposed NIA-AA criteria which state that amyloid pathology is required to be labeled as having AD pathophysiology but evidence of both Aβ and tau pathology is required in order to be labeled as having AD. The differentiation between CSF t-tau and p-tau could, however, be questioned as these markers are known to be highly correlated. The high number of resulting subgroup combinations may limit its applicability in clinical research settings.

While Aβ is considered the core pathology in preclinical AD, we still do not understand the pathophysiology of Aβ aggregation. To capture the earliest stages of AD, it is crucial to better understand the folding and aggregation of Aβ monomers into oligomers, protofibrils, and fibrils. Several studies have shown variability between individuals in type of aggregated Aβ but current assays may not be able to detect these different subtypes of aggregated Aβ. More knowledge on Aβ aggregation could shed new light on findings regarding individuals with slightly elevated levels of subthreshold Aβ who show cognitive decline [17, 60]. It can also help to understand the biomarker and cognitive trajectories of people with neuronal injury in the absence of Aβ pathology. Currently they are labeled as having Suspected Non-Alzheimer's disease pathophysiology (SNAP) [13], but a subgroup may as well have preclinical AD with a form of aggregated Aβ that cannot be picked up by current Aβ assays.

How preclinical AD relates to other AD-related molecular processes is another topic that requires further investigation. Synapse dysfunction, neuroinflammatory responses, and axonal degeneration are known to play a central role in AD, but exact timings are not yet fully understood [61, 62]. Knowledge on these processes in relation to core AD markers could help to identify subtypes of preclinical AD that may benefit from different treatments and improve prognosis in these individuals. Neurogranin [63], YKL-40 [64], and Neurofilament-Light [65] are relatively well-established novel CSF markers that reflect these processes and may be good biomarker candidates for prognosis. Large-scale multimodal omics studies, like the IMI EMIF-AD Biomarker Discovery Study (http://www.emif.eu/about/emif-ad), will contribute to the identification of novel genetic and molecular candidates to further refine preclinical AD.

A deeper understanding of the earliest cognitive changes in preclinical AD would be also of great importance for clinical research and AD trials. Studies have shown that composite measures of global cognition may best capture early cognitive changes in preclinical AD (see above) [24, 26]. However, most of the current tests were not developed for identifying AD in cognitively healthy individuals and cognitive norm scores are often based on a cognitively healthy population including also individuals

who have already amyloid pathology. Also the role of subjective cognitive complaints in relation to the earliest cognitive changes should be further clarified.

Key challenges of preclinical AD

Detection and diagnosis

The detection of Aβ pathology requires a lumbar puncture or PET scan. This complicates screening for preclinical AD among cognitively healthy individuals, as these tools are not yet widely used, more costly, and still considered relatively invasive. To implement screening for preclinical AD on a large scale there is a need for valid easily assessable biomarkers, like blood-based biomarkers. Validated blood-based markers are not yet available, but it is likely that in the future a combined set of blood-based and other markers can serve as signature for preclinical AD or as pre-selection tool for individuals that should undergo assessment of Aβ pathology by lumbar puncture or PET scan.

Without an available treatment, a diagnosis of AD in the preclinical stage comes with several ethical considerations. A preclinical AD diagnosis can only be considered in relation to clinical trial recruitment, and only with appropriate counseling. Preclinical AD should not be diagnosed in clinical routine as the prognosis is not clear, it may create stigma or induce worries in people who do not have clinical symptoms yet, and because there is no treatment. Nevertheless, there will be people who want to know their risk of progression to dementia. Shared decision-making will then be crucial such that persons understand what the findings can and what they cannot tell regarding diagnosis and prognosis.

Resilience and risk for progression

Not all individuals with preclinical AD will progress to dementia before death. Findings of post-mortem Aβ plaques in brains of cognitively healthy elderly at death raise the question why some of the individuals with preclinical AD are resilient for cognitive decline. Neuropathological studies suggested that those Aβ plaques appear to be associated with lower levels of oligomeric Aβ forms and could therefore be less toxic [66]. Also less neuroinflammation has been reported in brains of these individuals [67]. There are several environmental and lifestyle as well as genetic factors that can influence symptom expression in AD and prevent some individuals from becoming demented. As findings are still inconsistent about the protective role of healthy lifestyle on core AD pathology (see above), we need a better understanding of the molecular pathways by which cognitive, lifestyle, and genetic protective effects are exerted. Knowledge on factors that promote resilience could lead to novel therapeutic targets for individuals who are at high risk of progression to dementia. Understanding resilience in preclinical AD is also of utmost importance once a cure becomes available in order to avoid treating persons with preclinical AD who may never become demented.

Among individuals with preclinical AD who do progress to dementia there is a large variability in rate of progression. Disease progression may in part depend on the presence of other AD-related pathologies such as synapse dysfunction, neuroinflammation, and axonal degeneration. Preclinical AD manifests at older ages and therefore prognosis may also depend on the presence of age-related comorbid diseases, such as vascular pathology or vascular risk factors [68], which all influence the rate of cognitive decline. This indicates the need of a multidimensional approach to estimate the prognosis of preclinical AD.

Drug trial design

The concept of preclinical AD is very valuable for development of strategies to prevent cognitive impairment. It provides a large time window for disease-modifying treatment, as neurodegeneration is still limited. However, as it reflects a long early stage of the disease, clinically relevant cognitive changes cannot be easily captured. There is a need for cognitive tests that can monitor cognition over time in preclinical AD. Most of the current cognitive tests are rather developed for diagnostic purposes or capture only cognitive changes in more advanced stages of AD. Furthermore, it is not feasible to have treatment trials with a follow-up of more than 5 years. It is therefore essential to be able to translate small cognitive changes in preclinical AD to the expected clinical changes in daily functioning or quality of life in advanced stages of AD by statistical disease modeling. For example, the IMI ROADMAP project (https://roadmap-alzheimer.org) aims to develop disease and health-economic models to demonstrate long-term value of treatment in preclinical AD using data of population studies, clinical cohorts as well as EHR datasets that together cover the full AD clinical spectrum.

Timing is everything. Maybe AD can only be stopped before pathology arises such that the preclinical AD stage would already be too late to cure AD. Certain disease-modifying drugs, like drugs targeting

Aβ production and aggregation, are likely most effective before considerable Aβ accumulation has taken place. Primary prevention trials would need to test drugs in individuals who are at risk for AD but do not have AD pathology yet. To maximize efficacy in such trials, trial recruitment of cognitively normal individuals could then, besides *APOE* ε4 carriership, be enriched by family history, lifestyle, and vascular risk factors and absence of environmental or genetic factors that point toward resilience.

PRECLINICAL AD: THE FUTURE

The preclinical AD concept has proven to be of tremendous value in understanding AD pathophysiology in the earliest stages of the disease and has obviously advanced drug trials. However, there is still a lot more to learn about preclinical AD and its associated processes. Further refinement of the preclinical AD concept will help us to tackle the current challenges and foster further advancement in research, clinical trials, and eventually clinical practice. Once we move toward primary or secondary prevention of AD, we will be faced with new ethical considerations and challenges regarding detection and diagnosis in primary care settings. For now, it may be useful to promote a healthy lifestyle and treat vascular risk factors in cognitively healthy individuals, already in midlife.

DISCLOSURE STATEMENT

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Nutritional Intervention as a Preventive Approach for Cognitive-Related Outcomes in Cognitively Healthy Older Adults: A Systematic Review

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Abstract. The link diet-cognitive function/dementia has been largely investigated in observational studies; however, there was a lack of evidence from randomized clinical trials (RCTs) on the prevention of late-life cognitive disorders though dietary intervention in cognitively healthy older adults. In the present article, we systematically reviewed RCTs published in the last four years (2014–2017) exploring nutritional intervention efficacy in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged 60 years and older using different levels of investigation (i.e., dietary pattern changes/medical food/nutraceutical supplementation/multidomain approach and dietary macro- and micronutrient approaches) as well as possible underlying mechanisms of nutritional prevention. From the 35 included RCTs, there was moderate evidence that intervention through dietary pattern changes, medical food/nutraceutical supplementation, and multidomain approach improved specific cognitive domains or cognitive-related blood biomarkers. There was high evidence that protein supplementation improved specific cognitive domains or functional status in prefrail older adults without effect

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on cognitive function. For fatty acid supplementation, mainly long-chain polyunsaturated fatty acids, there was emerging evidence suggesting an impact of this approach in improving specific cognitive domains, magnetic resonance imaging (MRI) findings, and/or cognitive-related biomarkers also in selected subgroups of older subjects, although some results were conflicting. There was convincing evidence of an impact of non-flavonoid polyphenol and flavonoid supplementations in improving specific cognitive domains and/or MRI findings. Finally, there was only low evidence suggesting efficacy of intervention with homocysteine-related and antioxidant vitamins in improving cognitive functions, dementia incidence, or cognitive-related biomarkers in cognitively healthy older subjects.

Keywords: Alzheimer's disease, dementia, dietary pattern, healthy diet, macronutrients, medical food, Mediterranean diet, micronutrients, mild cognitive impairment, nutraceuticals, prevention

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by cognitive and behavioral impairment, significantly interfering with social and occupational functioning. It is an incurable progressive disease with a long presymptomatic period. In the United States, an estimated 5.4 million subjects have AD, and by mid-century, the number of people living with AD is projected to grow to 13.8 million [1]. Considering the absence of available disease-modifying therapies for the AD treatment [2], there is a great need in preventing and delaying the onset of cognitive impairment in healthy older subjects. In the last two decades, several observational studies have shown a wide variety of potentially modifiable risk factors for cognitive impairment [3], that have been proposed as targets for preventive strategies. In addition to cardiovascular risk factors, psychological conditions, education level, engagement in social and mentally stimulating activities, sensory changes, and lifestyle including diet, physical activity, and voluptuary habits has obtained a crucial role [3, 4]. In particular, in the last years, a growing body of evidence has been focused on the association between dietary habits and cognitive performance [4–7]. Several nutritional supplements have been studied for their potential role as neuroprotective interventions useful in delaying the onset of cognitive decline in older age. Observational studies have showed that specific micro/macronutrients such as polyunsaturated fatty acids (PUFAs), vitamins, and flavonoids were associated with a significantly reduced risk of dementia [8]. This protective effect could be mediated by several pathobiological pathways involved in AD development as amyloid-β (Aβ) deposition, neurofibrillary degeneration, synapse loss, inflammation, oxidative stress, mitochondrial dysfunction, loss of vascular integrity, and neuronal injury. Furthermore, in the last ten years, a growing body of epidemiological evidence suggested that foods and nutrients properly combined into specific dietary patterns may act synergistically amplifying the health effects of single components [4, 9–14]. The Mediterranean dietary pattern has been the first and widely well studied, showing a strong protective role in cardiovascular and cognitive aging [9–12]. Considering these promising results and the growing interest in this field, several randomized clinical trials (RCTs) investigating nutritional interventions as preventive or therapeutic approaches for cognitive-related outcomes have started obtaining contrasting results. Furthermore, in the last few years, the approach to the study of the diet-cognition relationship has been changed. In fact, according to the National Institute on Aging–Alzheimer's Association (NIA-AA) guidelines for AD and cognitive decline due to AD pathology [15], it has been suggested a direct impact of nutrition to brain structure and activity changes [4]. This consideration in addition to the need to objectively quantify the effects of nutrients on cognitive-related outcomes not only in terms of cognitive scores of clinical scales has opened the era of brain imaging biomarkers in nutritional epidemiology. Another feature to underline was the emerging use of objective measures of dietary habits, not only in terms of daily questionnaires, but also using biochemical markers (e.g., serum concentration or red blood cells levels) in order to achieve more reliable findings. The aim of the present study was to provide a comprehensive and updated systematic review focusing on the RCTs published in the past four years (2014–2017) exploring nutritional intervention efficacy in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years.

METHODS

In the present systematic review article, we followed the Preferred Reporting Items for Systematic

reviews and Meta-Analyses (PRISMA) guidelines, adhering to the PRISMA 27-item checklist [16].

This systematic review was based upon searches of US National Library of Medicine (PubMed), Ovid MEDLINE, EMBASE, Google Scholar, Web of Science, and Scopus databases, picking the following terms to identify the risk exposure (intervention OR supplementation AND dietary OR nutritional OR dietary patterns OR foods OR food groups OR micronutrients OR macronutrients OR medical foods OR nutraceuticals) combined with terms to determine the outcomes of interest [cognitive AND (impairment OR decline OR disorders) OR Alzheimer's disease OR dementia OR vascular dementia OR mild cognitive impairment]. There were no language restrictions on the search. To be included in this systematic review, studies were limited to RCTs published between January 1, 2014 and December 31, 2017.We choose these time limits on the basis of a pre-search without time limits that included a high number of identified articles to review (11,490 articles). Studies were further required to meet the following inclusion criteria: 1) studies conducted in cognitively healthy humans aged 60 years or older; 2) studies that provided a description of the tools used for collecting the adherence to the different dietary patterns and the intake of foods, food groups, micro- and macronutrients (e.g., validated semi-quantitative food frequency questionnaires, 3- and 7-day dietary records, or 24-h dietary recall) or that evaluated nutrient consumption from the values of biochemical markers (e.g., serum concentration or red blood cells levels); 3) studies providing the neuropsychological tools used for defining late-life cognitive impairment/decline also in nondemented and cognitively healthy older subjects. We excluded studies with diagnoses of mild cognitive impairment (MCI) [Petersen criteria and their revision/modifications, International Working Group on Mild Cognitive Impairment criteria, European Alzheimer's Disease Consortium (EADC) criteria, NIA-AA criteria for MCI due to AD, and DSM-5 criteria for Mild Neurocognitive Disorder), AD [National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria, NIA-AA criteria for dementia due to AD, International Working Group (IWG)-1 criteria for AD, and IWG-2 criteria for AD], prodromal AD (IWG-1 criteria for prodromal AD), preclinical states of AD (IWG-2 criteria for the preclinical states of AD), preclinical AD (NIA-AA criteria for preclinical AD), vascular dementia (VaD) (NINCDS-AIREN), and unspecified dementia [Diagnostic and Statistical Manual for Mental Disorders (DSM)-III-R criteria, DSM-IV criteria, DSM-IV-TR criteria, DSM-5 criteria for Major Neurocognitive Disorder, International Classification of Diseases (ICD), 9th Revision, Clinical Modification (CM), and ICD-10-CM]. The studies included had to present original data. Figure 1 shows the stages in obtaining studies for inclusion in the present report (PRISMA Four-phase Flow Diagram). From 2,528 articles identified with multiple electronic searches, we screened titles and abstracts of the citations downloaded from the searches and identified 992 potential relevant articles chosen for a closer review. We excluded 875 articles not meeting inclusion criteria and obtained full copies of the 117 potentially suitable reports for further assessment. After inclusion of 4 articles of interest from the reference lists of the selected articles and exclusion of another 82 articles, 35 studies met study eligibility criteria and were finally included in the overall systematic review [17–52] (Tables 1–3). We used the Risk of Bias Tool as recommended by the Cochrane Handbook to assess risk of bias in the included studies, which was assessed independently (VS, FP) on domains of random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias [53]. Furthermore, we used the GRADE approach to summarize overall quality of evidence the RCTs included in the present systematic review [54]. Finally, we used a narrative synthesis to summarize the findings of the included studies, subdividing the articles for the three principal diet-based approaches (dietary patterns/medical food/nutraceutical supplementation/multidomain approach, macronutrients, and micronutrients), specifying sample size and the cognitive outcomes of the included studies [17–52] (Tables 1–3).

NUTRITIONAL INTERVENTION THROUGH DIETARY PATTERN CHANGES, MEDICAL FOOD/NUTRACEUTICAL SUPPLEMENTATION AND MULTIDOMAIN APPROACH

Dietary pattern changes

Table 1 shows selected RCTs published in the last four years (2014-2017) that evaluated the efficacy

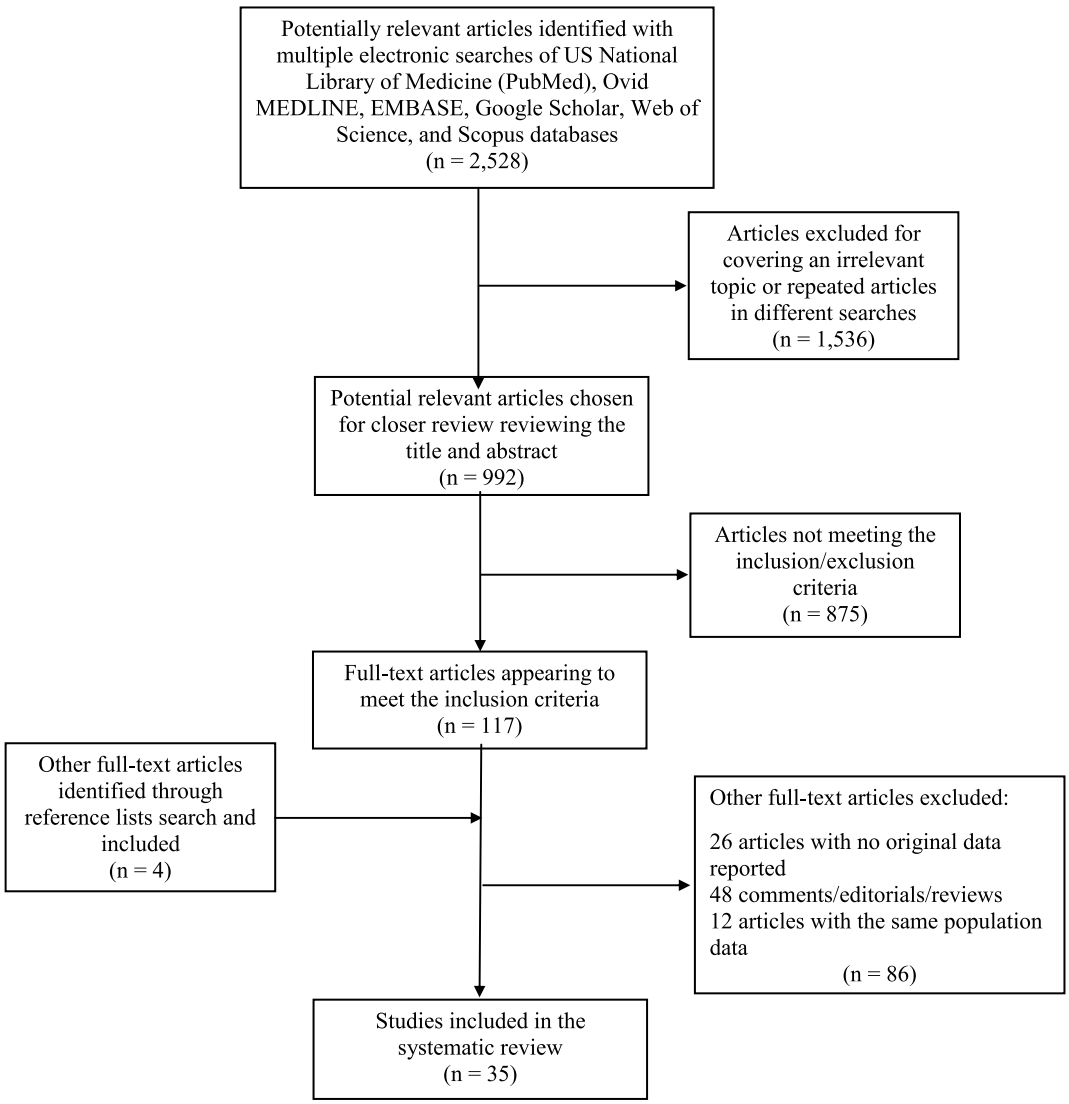

Fig. 1. PRISMA Four-phase Flow Diagram of retrieved and selected randomized clinical trials (RCTs) published in the past four years (2014–2017) exploring nutritional intervention efficacy in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years.

of nutritional intervention through dietary pattern changes, medical food/nutraceutical supplementation, and multidomain approach in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years [17–25]. A growing body of epidemiological evidence suggested the importance to consider not only the total caloric intake but also the quality of macro-/micronutrients properly combined. It is doubtless that the Mediterranean diet (MeDi), the typical dietary pattern of Mediterranean countries, has been the most studied dietary pattern and proposed to have a protective role against cognitive decline and dementia. The main components of the MeDi pattern are fruits, vegetables, legumes, cereals, and olive oil as the main added lipid, associated with a moderate consumption of red wine and low consumption of red meat and dairy products. In particular, the findings from prospective observational studies and very recent systematic reviews and meta-analyses of pooled studies suggested that higher adherence to the MeDi fulfilling the whole-diet approach was associated with a reduced risk of cognitive impairment, MCI and AD, as well as the transition from MCI to AD [4, 9–11]. Moreover, a recent systematic review on this issue suggested that also other

Table 1
Randomized clinical trials evaluating the efficacy of nutritional intervention through dietary pattern changes, medical food/nutraceutical supplementation, and multidomain approach in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years (2014–2017)

Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Principal results
DIETARY PATTERN CHANGES					
Valls-Pedret et al., 2015 [17]	447 cognitively healthy older subjects Mean age: 68.2 ± 6.3 years for the intervention group and 68.8 ± 6.5 years for the placebo group	• MeDi+ EVOO (1L/week) • MeDi+ mixed nuts (30g/day) • Control diet (advice to reduce dietary fat)	4.1 years (median)	MMSE, AVLT, ASF, DS-WAIS, VPA-WAIS, CTT	In an older population, a MeDi supplemented with EVOO or nuts was associated with improved episodic memory and frontal and global cognition
Assaf et al., 2016 [18]	48,835 older women Aged: 50–79 years	• Intervention group: reduced calories from fat to 20%, increased vegetables and fruit to 5 + servings, and increased grain servings to 6 + servings a day • Placebo	8.1 + 1.7 y (max.11.2 y)	3MSE, RAND36 WHI FFQ.	No significant improvement in cognitive functions. Small significant improvements in three health-related quality of life subscales: general health, physical functioning, and vitality at 1-year follow-up
MEDICAL FOOD/NUTRACEUTICAL SUPPLEMENTATION					
Small et al., 2014 [19]	105 cognitively intact adults Aged: 65–85 years	• Nutraceutical NT-020 • Placebo	8 weeks	MMSE, AVLT, IPT, NC, TMT-A and –B, FBDS-WAIS, CF, COWAT, DST	Better performance for the NT-020 group in two measures of processing speed (IPT and NC) compared to placebo group
Lewis et al., 2014 [20]	97 cognitively healthy older subjects MMSE ≥ 23 Aged ≥ 60 years	• Ginkgo Synergy® plus Choline • OPC Synergy® plus Catalyn® • Placebo	6 months	MMSE, SCWT, TMT-A and –B, COWAT, DS-WAIS-III, HVLT-R Immune function markers	Isolated and modest effects of a Ginkgo biloba plus choline-based formula on cognitive (executive functioning and verbal fluency) and immune functioning among healthy older adults with no history of significant cognitive deficits
Harris et al., 2015 [21]	116 healthy older participants Aged: 55–65 years	• Multivitamin, mineral and herbal supplements • Placebo	16 weeks	CRT, IDRM, SI, SWM, and CM, blood biomarkers relevant to cognition	In cognitively healthy older people, multivitamin supplementation improved a number of cognitive-related blood biomarkers, but these biomarker changes were not accompanied by no significant improvement in cognitive functions

(*Continued*)

Table 1
(*Continued*)

Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Principal results
Strike et al., 2016 [22]	27 postmenopausal women Aged: 60–84 years	• Efalex Active 50+ • Placebo	6 months	MOT, VRM, and PAL, mobility was assessed by VICON 9 motion capture camera system synchronized with Kistler force plates; blood fatty acid levels by pin-prick analysis	In this RCT, multinutrient supplementation improved cognition and mobility in healthy older females suggesting a potential role in reducing the decline to frailty.
MULTIDOMAIN APPROACH					
Van de Rest et al., 2014 [23]	127 frail or pre-frail older subjects Mean age: 79 years	• Protein (30 g/day) • Protein + physical exercise • Placebo • Placebo + physical exercise	24 weeks	MMSE, TMT-A and –B, WLT, SCWT, FBDS-WAIS, VFT, and reaction time tasks 3-day dietary record	Significant improvement of information processing speed in the protein plus physical exercise group
Ngandu et al., 2015 [24]	1,260 nondemented older subjects Aged: 60–77 years	• Multidomain lifestyle intervention • Control group	2 years	A comprehensive NTB Z score	Findings from this long-term, RCT suggested that a multidomain intervention could improve or maintain cognitive functioning in at-risk older people
Andrieu et al., 2017 [25]	1,680 nondemented older subjects Aged: 70 years or older	• Multidomain intervention plus n-3 PUFAs) • Multidomain intervention plus placebo, • n-3 PUFAs alone • Placebo alone	3 years	Z score combining free and total recall of the FCSRT, ten MMSE orientation items, DSST, and CNT	The multidomain intervention and n-3 PUFAs, either alone or in combination, had no significant effects on cognitive decline over 3 years in older people with memory complaints

MeDi, Mediterranean diet; EVOO, extra virgin olive oil; MMSE: Mini-Mental State Examination; AVLT: Rey Auditory Verbal Learning Test; ASF: Animals Semantic Fluency; DS-WAIS, Digit Span subtest from the Wechsler Adult Intelligence Scale; VPA-WAIS: Verbal Paired Associates from the Wechsler Memory Scale; CTT, Color Trail Test; 3MSE, modified Mini-Mental State Examination; RAND36, RAND 36-Item Health Survey; WHI, Women's Health Initiative; FFQ, food frequency questionnaires; IPT, Identical Pictures Test; NC, Number Comparison task; TMT-A, Trail Making Test - A; TMT-B, Trail Making Test -B; FBDS-WAIS, Forward and Backward Digit Span task; CF, Category Fluency; COWAT, Controlled Oral Word Association Test; DST, Digit Symbol Tests; SCWT, Stroop Color-Word Test; DS-WAIS-III, Digit Symbol subtest from the Wechsler Adult Intelligence Scale- III; HVLT-R, the Hopkins Verbal Learning Test-Revised; CRT, Choice Reaction Time; IDRM, Immediate and Delayed Recognition Memory; SI, Stroop Interference tasks; SWM, Spatial Working Memory; CM, Contextual Memory; MOT, psychomotor response latency; VRM, Verbal Recognition Memory; PAL, paired associate learning; WLT, Word Learning Test, SCWT: Stroop Color-Word Test, VFT, Verbal Fluency Test; PUFAs, polyunsaturated fatty acids; NTB, neuropsychological test battery; FCSRT, Free and Cued Selective Reminding test; DSST, Digit Symbol Substitution Test; CNT, Category Naming Test.

emerging healthy dietary patterns such as the Dietary Approach to Stop Hypertension (DASH) and the Mediterranean-DASH diet Intervention for Neurodegenerative Delay (MIND) diets were associated with slower rates of cognitive decline and significant reduction of AD rate [4]. Despite observational studies showed a positive significant association of certain healthy dietary patterns with cognitive impairment, only few interventional studies have been conducted on dietary patterns, particularly on MeDi, reporting contrasting findings. In fact, in an RCT including 447 cognitively normal participants randomly assigned to a MeDi supplemented with extra virgin olive oil (EVOO) or with mixed nuts, or a control diet for a 4.1 years follow-up, those allocated to a MeDi plus EVOO scored better on the

Table 2
Randomized clinical trials evaluating the efficacy of nutritional intervention using a macronutrient approach in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years (2014–2017)

Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Main Results Cognitive results
PROTEIN SUPPLEMENTATION					
Van der Zwaluw et al., 2014 [26]	65 frail or pre-frail older subjects Mean age: 79 years	• Proteins (30 g/day) • Placebo	24 weeks	MMSE, TMT-A and –B, WLT, SCWT, FBDS-WAIS, VFT, and reaction time tasks 3-day dietary record	Protein supplementation improved reaction time performance in pre-frail and frail older adults, but did not improve other cognitive functions
Szcześniak et al., 2014 [27]	51 older subjects MMSE≥15 Mean age: 81 ± 7 years in CRC group and 80.5 ± 7.5 years in placebo group	• CME containing 40% of CRC (2:1 ratio of anserine to carnosine) was administered 2.5g/day • Placebo	13 weeks	MMSE, STMS, and CDR	A significant improvement was found after supplementation in specific subscores of STMS, a test evaluating global cognitive functions, such as construction/copying, abstraction, and recall
Rokicki et al., 2015 [28]	31 cognitively healthy participants Aged: 42–78 years	• Twicedaily doses of the imidazole dipeptide formula with 500 mg of CRC in total • Placebo	3 months	ADAScog, WMSLM 1 and 2, and BDI Functional MRI	In the CRC group, better verbal episodic memory performance and decreased connectivity on functional MRI were found
Hisatsune et al., 2016 [29]	39 cognitively healthy participants Mean age: 69.2 years	• Twice-daily doses of the imidazole dipeptide formula with 500 mg of CRC in total • Placebo	3 months	ADAScog, WMSLM 1 and 2, BDI, SF-36, MMSE Serum concentrations of 27 cytokines Perfusion MRI	CRC supplementation showed a significant beneficial effect on verbal episodic memory and brain perfusion in older adults
Badrasawi et al., 2016 [30]	50 pre-frail cognitively healthy participants Mean age: 68.2 ± 6.3 years for the intervention group and 68.8 ± 6.5 years for the placebo group	• L-carnitine supplementation (500 mg/cap) • Placebo	10 weeks	MMSE, physical frailty status, FI, PASE Selected frailty biomarkers Anthropometric measurements	L-carnitine supplementation had a favorable effect on the functional status and fatigue in prefrail older adults, without effect on nutritional status, body composition, and cognitive function

(*Continued*)

Table 2
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Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Main Results Cognitive results
FATTY ACID SUPPLEMENTATION					
Witte et al., 2014 [31]	65 cognitively healthy older subjects MMSE≥26 Mean Age: 63.9 ± 6.6 years	• n-3 PUFA group received fish oil capsules with 2.2 g of n-3 PUFAs (1320 mg EPA + 880 mg DHA, given as 1000 mg fish oil and 15 mg vitamin E) • Placebo (sunflower oil)	26 weeks	VF, TMT-A and –B, SCWT, AVLT, FBDS-WAIS, STAI 1 and 2 Erythrocyte membrane fatty acid composition MRI	Supplementation with high levels of n-3-PUFAs demonstrated enhanced executive functions in healthy older adults after 26 weeks and improved white matter microstructural integrity, regional gray matter volume, and vascular parameters
Jaremka et al., 2014 [32]	138 cognitively healthy older subjects Mean age: 51.0 ± 7.8 years	• 1.25 g/day of n-3 PUFAs • 2.50 g/day of n-3 PUFAs • Placebo The fish oil supplements contained a 7:1 ratio of EPA to DHA	4 months	20-item UCLA loneliness scale, CVLT –II, DS-WMS-III, LNS-WMS-III, SS-WMS-III, TMT, COWAT Plasma levels of n-6 and n-3 PUFAs.	Lonelier people within the placebo condition had poorer verbal episodic memory post-supplementation, as measured by immediate and long-delay free recall, than their less lonely counterparts. The plasma n-6 PUFAs:n-3 PUFAs ratio data mirrored these results
Mahmoudi et al., 2014 [33]	199 older individuals with normal or mild to moderate cognition impairment Aged ≥ 65 years	• 180 mg of DHA + 120 mg of EPA • Placebo	6 months	MMSE, AMT Plasma cholesterol, CRP, fasting blood sugar	No significant effects on cognitive outcomes
Chew et al., 2015 [34]	3073 participants Mean Age: 72.7 years	• n-3 PUFAs (1g) and/or lutein (10mg)/zeaxanthin (2mg) • Placebo All participants were also given varying combinations of vitamins C, E, beta carotene, and zinc	5 years	HHI, CES-D, TICS-M, TICS-M Recall, AC, LF, AF, LM-WMS-III-1 & 2, DB, and DR-WMS-III-RP	No significant effects on cognitive outcomes
Pase et al., 2015 [35]	160 cognitively healthy older volunteers Aged: 50–70 years	• Multivitamin combined with Þsh oil (3 g) • Multivitamin combined with Þsh oil (6 g) • Placebo multivitamin combined with Þsh oil (6 g) • Placebo multivitamin combined with placebo Þsh oil (Sunola oil)	16 weeks	SUCCAB measuring reaction time, cognitive processing speed, short-term memory, and visual memory BP variables	Absolute increases in the red blood cell n-3/n-6 ratio were associated with improvements in spatial working memory. The 6 g fish oil without the multivitamin group displayed a significant decrease in aortic pulse pressure and aortic augmentation pressure, two measures of aortic BP and aortic stiffness

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Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Main Results Cognitive results
Tokuda et al., 2015 [36]	113 older nondemented Japanese men Mean age: 59.6 years	• LC-PUFA-containing oil (including ARA 120 mg/die, DHA 300 mg/die and EPA 100 mg/die) • Purified olive oil	4 weeks	Event-related potential P300 and POMS LC PUFA plasma analysis Diet history questionnaire, semi-quantitative FFQ, and study diary	Changes in P300 latency were significantly different between the placebo group and the LC PUFA group after supplementation
Külzow et al., 2016 [37]	44 cognitively healthy individuals Mean age: 62 ± 6 years	• n-3 PUFAs (2.2g/day) • Placebo	26 weeks	LOCATO assessing OLM in older adults, AVLT, and PANAS Erythrocyte membrane fatty acid composition, serum biomarkers and APOE genotyping Dietary habit questionnaire	Performance in cued recall in a OLM task was sensitive in detecting beneficial effects of n-3 PUFA supplementation. Omega-3-index significantly increased in the n-3 PUFA group and decreased in the placebo group
Jackson et al., 2016 [38]	86 cognitively healthy individuals who reported subjective memory deficits Aged: 50–70 years	• DHA-rich fish oil 2 g (896 mg DHA, 128 mg EPA) • Efalex Active 50 + containing 2 g DHA-rich fish oil (946.4 mg DHA, 160 mg EPA) plus phosphatidylserine (88 mg), Ginkgo biloba (240 mg), folic acid (1 mg) and vitamin B12 (24 mg) • Placebo	6 months	CDB LC PUFA plasma analysis Functional near infrared spectroscopy (NIRS)	The findings from this RCT indicated no effect of either the multinutrient supplement or DHA-rich fish oil on either the NIRS or cognitive outcomes
Mazereeuw et al., 2016 [39]	92 cognitively healthy subjects with CAD Aged: 45–80 years	• n-3 PUFAs (1.9 g/day) • Placebo	12 weeks	HAM-D, BDI-II, and NINDS/CSN neuropsychological battery for vascular cognitive impairment n-3 PUFA plasma analysis	Treatment did not improve cognitive performance; however, n-3 PUFAs significantly increased verbal memory compared with placebo in a subgroup of nondepressed patients

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Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Main Results Cognitive results
Boespflug et al., 2016 [40]	21 cognitively healthy older adults with subjective memory impairment, but not meeting criteria for MCI or dementia Aged: 62–80 years	• Fish oil (EPA+DHA, 2.4 g/day) • Placebo (corn oil)	24 weeks	Cortical blood oxygen level-dependent (BOLD) activity during a working memory task by functional MRI Erythrocyte membrane fatty acid composition	Dietary fish oil supplementation increased red blood cell n-3 PUFA content, working memory performance, and BOLD signal in the posterior cingulate cortex during greater working memory load in older adults with subjective memory impairment

MMSE, Mini-Mental State Examination; TMT-A, Trail Making Test - A; TMT-B, Trail Making Test -B; WLT, Word Learning Test; SCWT, Stroop Color-Word Test; FBDS-WAIS, Forward and Backward Digit Span task from the Wechsler Adult Intelligence Scale; VFT, Verbal Fluency Test; CRC, carnosine related compounds; CME, chicken meat extract; STMS, Short Test of Mental Status; CDR, Clinical Dementia Rating; ADAS-cog, Alzheimer's Disease Assessment Scale-Cognition; LM-1 & 2-WMS-, Logical Memory 1 & 2 from Wechsler Memory Scale; BDI, Beck Depression Inventory; MRI, magnetic resonance imaging; SF-36, Medical Outcomes Study, 36-item Short Form; MCS, Mental Health Component Summary score; PCS, Physical Health Component Summary; FI, Frailty Index; PASE, Physical Activity Scale for Elderly; n-3 PUFA, n-3 polyunsaturated fatty acids; VF, Verbal Fluency; AVLT, Auditory Verbal Learning Test; STAI 1 and 2, Spielberger's State-Trait Angst Inventar; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; CVLT, California Verbal Learning Test, Second Edition; DS-WMS-III, Digit Span subtest from the Wechsler Memory Scale – Third Edition; LNS-WMS-III, Letter-Number Sequencing subtest from the Wechsler Memory Scale – Third Edition; SS-WMS-III, Spatial Span subtest from the Wechsler Memory Scale – Third Edition; COWAT, Controlled Oral Word Association Test; AMT, Abbreviated Mental Test score; CRP, C-reactive protein; CES-D, Center for Epidemiologic Studies' Depression Scale; TICS-M, Telephone Interview Cognitive Status-Modified; AC, Animal Category; LF, Letter Fluency; AF, Alternating Fluency; LM-WMS-III-1 & 2, Logical Memory 1 & 2 from Wechsler Memory Scale – Third Edition; DB, Digits Backward; DR-WMS-III-RP, Delayed Recall from Wechsler Memory Scale – Third Edition Recall Paragraph; SUCCAB, Swinburne University Computerised Cognitive Assessment Battery; BP, blood pressure; LC PUFA, long-chain polyunsaturated fatty acids; ARA, arachidonic acid; POMS, Profile of Mood Status; FFQ, food frequency questionnaires; OLM, object-location memory; PANAS, Positive and Negative Affect Schedule; APOE, apolipoprotein E; CDB, Cognitive Demand Battery; NIRS, near infrared spectroscopy; CAD, coronary artery disease; HAM-D, Hamilton Depression Rating Scale; BDI-II, Beck Depression Inventory II; NINDS/CSN, National Institutes of Neurological Disorders and Stroke and Canadian Stroke Network; MCI, mild cognitive impairment; BOLD, blood oxygen level-dependent.

episodic memory and attention tasks compared with the control group. Furthermore, compared with controls, this RCT showed a significant improvement in memory composite in the MeDi plus nuts group and a significant improvement in frontal and global cognition composites in the MeDi plus EVOO group [17] (Table 1). Furthermore, in a large RCT recruiting 48,835 women (50–79 years) for a follow-up of mean length of 8.1 years, dietary intervention based on caloric fat restriction and increasing consumption of vegetables, fruit, and grain had no significant effects on cognition, with small significant improvements in three health-related quality of life subscales: general health, physical functioning, and vitality at one year follow-up [18] (Table 1).

Medical food/nutraceutical supplementation

In the last decade, several RCTs have proposed medical foods/nutraceuticals as preventive or therapeutic approaches for cognitive decline and dementia, according to the increasing knowledge about the potential beneficial effect of specific nutrients properly combined in selected dietary patterns [55]. In the last four years, some medical foods/nutraceuticals have been tested in cognitively healthy subjects in order to delay cognitive impairment obtaining good results only in specific cognitive domains [19–22] (Table 1). In a RCT, 105 cognitively intact adults were randomized to receive a pill-based nutraceutical (NT-020), a proprietary formulation of blueberry, green tea extract (95% polyphenols), carnosine, VitaBlue (40% polyphenolics, 12.5% anthocyanins from blueberries), and vitamin D3 (2000 IU per serving) and also contains grape polyphenolics, including 5% resveratrol (40 mg Biovin) or placebo using a battery of neuropsychological tests assessing six broad cognitive domains (episodic memory, processing speed, verbal ability, working memory, executive functioning, and

Table 3
Randomized clinical trials evaluating the efficacy of nutritional intervention using a micronutrient approach in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years (2014–2017)

Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Main Results
NON-FLAVONOID POLYPHENOLS					
Witte et al., 2014 [41]	46 cognitively healthy overweight older individuals Aged: 50–75 years	• Daily intake of four capsules (in total 200 mg of resveratrol and 320 mg of quercetin) • Placebo All subjects received a 13week supply of capsules and another 13-week supply after 3 months.	26 weeks	AVLT Functional MRI and DTI MRI Lipid metabolism, inflammation, neurotrophic factors, and vascular parameters	Significant positive effect of resveratrol on retention of words over 30 minutes and functional connectivity of the hippocampus with frontal, parietal, and occipital areas in healthy older overweight adults compared with placebo
Evans et al., 2017 [42]	80 post-menopausal women Aged: 45–85 years	• Resveratrol supplementation (75 mg twice daily) • Placebo	14 weeks	AVLT, CSMB, DSST, TMT, POMS, and CES-D TCD ultrasound CVR to both cognitive testing and hypercapnia	Compared to placebo, resveratrol elicited 17% increases in CVR to both hypercapnic and cognitive stimuli. Significant improvements were observed in the performance of cognitive tasks in the domain of verbal memory and in overall cognitive performance, which correlated with the increase in CVR
Rainey-Smith et al., 2016 [43]	96 community-dwelling older adults without significant cognitive impairments Mean age: 66 + 6.6 years	• 1500mg/day Biocurcumax™ • Placebo	12 months	CCRT, DASS, SF-36, PRMQ-16, MoCA; AVLT, COWAT, WDSS-WAIS-R, and the computerized CogState battery APOE genotyping	A significant time × treatment group interaction for global cognition, explained by a function decline in the placebo group at 6 months that was not found in the intervention group. No differences for all other clinical and cognitive measures
FLAVONOIDS					
Brickman et al., 2014 [44]	37 cognitively healthy, sedentary older subjects Aged: 50–69 years	• High flavanol intake + aerobic exercise • High flavanol intake Low flavanol intake + aerobic exercise • Low flavanol intake	12 weeks	ModBent task, AVLT Functional MRI	High dietary flavanol consumption enhanced dentate gyrus function in the aging human hippocampal circuit, independently of exercise

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Table 3
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Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Main Results
St John et al., 2014 [45]	300 cognitively healthy women Mean age: 61 years	• 25 g of isoflavone-rich soy protein (91 mg of aglycone weight isoflavones: 52 mg genistein, 36 mg daidzein, and 3 mg glycitein) • Milk protein-matched placebo provided daily	2.7 years	WTAR, CES-D; neuropsychological test battery evaluating general intelligence (executive/ expressive/ visuospatial tasks), verbal episodic memory (list learning/ logical memory), and visual episodic memory Overnight urine excretion of isoflavonoids and fasting plasma levels of isoflavonoids	Long-term changes in isoflavonoids were not associated with global cognition. Increasing isoflavonoid exposure from dietary supplements was, however, associated with decrements in general intelligence but not memory
Kean et al. 2015 [46]	37 cognitively healthy older subjects Mean age: 66.7 years	• High flavanone drink (305 mg/day) • Low flavanone drink (37 mg/day)	8 weeks	CERAD immediate and delayed verbal recalls and serial sevens, SWM, DSST-WAIS, VPA-WMS-III, LM, LF, and Go-NoGo	Daily consumption of high dose flavanone-rich orange juice was associated with benefits for global cognitive function, executive function, and episodic memory, mainly immediate recall
Mastroiacovo et al. 2015 [47]	90 cognitively healthy older subjects Mean age: 69.5 years	• 993 mg flavanols/day • 520 mg flavanols/day • 48 mg flavanols/day	8 weeks	MMSE, TMT-A and –B, and VFT	High dose flavanol consumption caused significant effects on executive function and verbal fluency
Nilsson et al., 2017 [48]	40 cognitively healthy older subjects Aged: 50–70 years	• Daily intake of mixed berry beverage (150 g blueberries, 50 g blackcurrant, 50 g elderberry, 50 g lingonberries, 50 g strawberry, and 100 g tomatoes) • Placebo	5 weeks	VWMT and SAT Cardiometabolic risk markers	Subjects performed better in the working memory domain after the berry beverage compared to the control beverage
HOMOCYSTEINE-RELATED AND ANTIOXIDANT VITAMINS					
Van der Zwaluw et al., 2014 [49]	2.919 older participants with Hcy levels between 12 and 50μmol/L Mean age: 74.1 + 6.5 years	• Daily either a tablet with 400 μg folic acid and 500 μg vitamin B12 • Placebo Both tablets contained 15μg vitamin D3	2 years	MMSE, AVLT, FBDS-WAIS, TMT-A and –B, SCWT, SDMT, and LF Blood biomarkers	This large RCT did not reveal beneficial effects of supplementation with vitamin B12 and folic acid on the cognitive domains of episodic memory, attention and working memory, information processing speed, and executive function

Table 3
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Reference	Study Sample	Intervention(s)	Duration	Cognitive-related outcomes and nutritional assessment	Main Results
Dangour et al., 2015 [50]	201 older subjects with moderate vitamin B-12 deficiency (serum vitamin B-12 concentrations: 107-210pmol/L) in the absence of anemia Mean age: 80 years	• 1 mg crystalline vitamin B-12 • Placebo	12 months	CVLT, SLMT, simple and choice reaction time, and VFT Peripheral motor and sensory nerve conduction and central motor conduction assessment	No evidence of an effect on peripheral nerve or central motor function outcome or on cognitive function
Cheng et al., 2016 [51]	104 older participants with hyperhomocysteinemia Mean age: 71.7 ± 8.8 years	• Vitamin B group, which received 800 μg/day of folate, with 10 mg of vitamin B6 and 25 μg of vitamin B12 • Placebo	14 weeks	BCATs Serum measure of tHcy, vitamin B6, vitamin B12, and folate	Improvement with vitamin B supplementation in global cognitive scores and four subtests (mental speed, visuo-spatial ability, working memory, and visual memory)
Kryscio et al., 2017 [52]	7.540 older men without cognitive impairment Aged: 60 years and older	• Vitamin E (400 IU/day) plus selenium (200 μg/day) • Vitamin E (400 IU/day) • Selenium (200 μg/day) • Placebo	5.4 years	Dementia case ascertainment	Dementia incidence (4.43%) was not different among the four study arms

AVLT, Auditory Verbal Learning Test; DTI, diffusion tensor imaging; MRI, magnetic resonance imaging; CSMB, Cambridge Semantic Memory Battery; DSST, Double Span Task; TMT, Trail Making Test; POMS, Profile of Mood Status; CES-D, Centre for Epidemiologic Studies Depression scale; TCD, Transcranial Doppler; CVR, cerebrovascular responsiveness; CCRT, Cambridge Contextual Reading Test; DASS, Depression Anxiety Stress Scales; PRMQ-16, 16 item self-report Prospective and Retrospective Memory Questionnaire; MoCA, Montreal Cognitive Assessment; WDSS-WAIS-R, Wechsler Digit Symbol Scale from Wechsler Adult Intelligence Scale revised; APOE, apolipoprotein E; Mod Bent; modified Benton Visual Retention Test; WTAR, Wechsler Test of Adult Reading; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; SWM, Spatial Working Memory; DSST-WAIS, Digit Symbol Substitution Test from the Wechsler Adult Intelligence Scale; VPA-WMS-III, Verbal Paired Associates from the Wechsler Memory Scale– Third Edition; LM, Letter Memory; LF, Letter Fluency; TMT-A, Trail Making Test - A; TMT-B, Trail Making Test -B; VFT, Verbal Fluency Test; VWMT, verbal working memory test; SAT, selective attention test; Hcy, homocysteine; FBDS-WAIS, Forward and Backward Digit Span task from the Wechsler Adult Intelligence Scale; SCWT, Stroop Color-Word Test; SDMT, Symbol Digit Modalities Test; CVLT, California Verbal Learning Test, Second Edition; SLMT, symbol letter modality test; BCATs, Basic Cognitive Aptitude Tests.

complex speed) at baseline and eight weeks later [19]. The NT-020 group exhibited better performance on two measures of processing speed than the placebo group at eight weeks of follow-up [19] (Table 1). Among nutraceutical compounds and combinatorial formulations, Ginkgo biloba extract is probably the most widely studied and used herbal-based medication for the prevention and treatment of AD and late-life cognitive decline [56]. Notwithstanding negative meta-analytic findings and the discouraging results of preventive trials against AD, some RCTs focusing particularly on dementia, AD, and MCI subgroups with neuropsychiatric symptoms and some recent meta-analyses have suggested a renowned role for Ginkgo biloba extract for cognitive impairment and dementia [56]. An RCT on 97 cognitively healthy older adults with no history of significant cognitive deficits reported modest effects of Ginkgo biloba plus choline-based formula on specific cognitive domains (executive functioning and verbal fluency) and immune functioning [20] (Table 1). An interesting RCT including 116 healthy cognitively older participants investigated the effects of supplementation with two multivitamin, mineral and herbal supplements, a women's formula and a men's formula in women and men, respectively. Assessments at baseline and post-supplementation included computerized cognitive tasks and blood biomarkers relevant to cognitive aging. After 16 weeks of follow-up, no cognitive improvements were observed after supplementation with either formula, while several significant improvements were observed in cognitive-related blood biomarkers including increased levels of vitamins B6 and B12 in women and men,

reduced C-reactive protein in women, reduced homocysteine (Hcy) and marginally reduced oxidative stress in men, as well as improvements to the lipid profile in men [21] (Table 1). Finally, in one RCT, 27 postmenopausal women received either Efalex Active 50 + [1 g docosahexaenoic acid (DHA), 160 mg eicosapentaenoic acid (EPA), 240 mg Ginkgo biloba, 60 mg phosphatidylserine, 20 mg d-α tocopherol, 1 mg folic acid, and 20 μg vitamin B12 per day] or placebo for 6 months. The intervention resulted in significant effects in two of the four cognitive tests, with shorter mean latencies in a motor screening task, and more words remembered, and one of the three primary mobility measures with improved habitual walking speed. Compared with the placebo group, supplementation also resulted in significantly higher blood DHA levels [22] (Table 1).

Multidomain approach

Considering the great interest on the relationships between a healthy lifestyle including optimal dietary habits and physical activity and an healthy cognitive aging, some studies have proposed a multidomain approach as an effective preventive approach for cognitive impairment or dementia [23–25] (Table 1). The findings of several RCTs have suggested that some single-domain interventions, i.e., antihypertensives, nutritional supplements, cognitive training, and physical activity, had protective effects on cognitive decline [57], but these results have seldom been replicated in larger samples. In two 24-week RCTs carried out in parallel, 127 older subjects performed a resistance-type physical exercise program or not and, in both studies, subjects were randomly allocated to either a protein drink (2×15 g daily) or a placebo one. In frail and pre-frail older adults, resistance-type exercise training combined with protein supplementation significantly improved information processing speed, whereas exercise training alone had significant good effects on attention and working memory. There were no significant differences among the intervention groups on the other cognitive tests or domain scores [23] (Table 1). Finally, in 2015, a successful 2-year multi-domain lifestyle intervention was completed aiming at prevention of cognitive decline, the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) [24] (Table 1), with dietary counselling as one of the intervention domains (diet, exercise, cognitive training, vascular risk monitoring) and a control group (general health advice). Intervention goals were based on Finnish dietary recommendations. This 2-year multidomain lifestyle intervention was conducted on 631 participants in the intervention and 629 in the control group, aged 60–77 years at baseline with an estimated mean change in neuropsychological test battery total Z score at 2 years of 0.2 in the intervention group and 0.16 in the control group. These findings from the FINGER suggested that a multidomain intervention could improve or maintain cognitive functioning in at-risk older people from the general population [24] (Table 1). Finally, the Multidomain Alzheimer Preventive Trial (MAPT) was a 3-year, multicenter, RCT with four parallel groups at 13 memory centers in France and Monaco. Participants were nondemented, aged 70 years or older, and community-dwelling, and had either relayed a spontaneous memory complaint to their physician, limitations in one instrumental activity of daily living, or slow gait speed. They were randomly assigned to either the multidomain intervention (43 group sessions integrating cognitive training, physical activity, and nutrition, and three preventive consultations) plus n-3 PUFAs (i.e., two capsules a day providing a total daily dose of 800 mg DHA and 225 mg EPA), the multidomain intervention plus placebo, n-3 PUFAs alone, or placebo alone [25] (Table 1). In the MAPT, a multidomain lifestyle intervention and n-3 PUFAs, either individually or in combination, did not significantly reduce cognitive decline over 3 years compared with placebo. The results of exploratory subgroup analyses suggested that the combined n-3 PUFA and multidomain intervention or the multidomain intervention alone might help to slow cognitive decline in people most likely to undergo cognitive decline, i.e., those with a Cardiovascular Risk Factors, Aging, and Incidence of Dementia (CAIDE) dementia risk score of 6 or greater at baseline, and those with a positive amyloid positron emission tomography (PET) scan [25] (Table 1).

Summary of evidence

Among 9 selected RCTs published in the last four years that evaluated the efficacy of nutritional intervention through dietary pattern changes, medical food/nutraceutical supplementation, and multidomain approach in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years [17–25], while 7 RCTs suggested an impact of these approaches in improving specific cognitive domains [17, 19, 20, 22–24] or only cognitive-related blood biomark-

ers with no significant improvement in cognitive functions [21], 2 RCTs did not find significant improvement in cognitive functions after dietary pattern changes [18] or multidomain intervention [25].

NUTRITIONAL INTERVENTION THROUGH MACRO- AND MICRONUTRIENT CHANGES

Macronutrients

Table 2 shows selected RCTs published in the last four years (2014–2017) that evaluated the efficacy of nutritional intervention through supplementation of dietary macronutrients in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years [26–40]. In particular, many interventional RCTs evaluated the cognitive impact of macronutrient intakes such as proteins and PUFAs with promising results.

Proteins

Many RCTs evaluated protein intake as a supplementation in nondemented older adults showing significant improvement in specific cognitive domains [26–30] (Table 2). Interestingly, these RCTs reported also promising results not only in terms of cognitive outcomes, but also magnetic resonance imaging (MRI) findings. In one RCT on 65 frail or pre-frail cognitively healthy older subjects randomly assigned to protein drink or placebo for 24 weeks, protein supplementation improved reaction time performance, but did not improve the cognitive domains of episodic memory, attention and working memory, information processing speed, and executive functioning [26] (Table 2). Promising results have been reported in a trial including 51 cognitively healthy older subjects [Mini-Mental State Examination (MMSE) >15] randomly assigned to dietary carnosine and anserine (carnosine related compounds, CRC) supplementation (chicken meat extract) or placebo. In this trial, a significant improvement after supplementation was found in specific subscores of a test evaluating global cognitive functions, such as construction/copying, abstraction, and recall [27] (Table 2). After 3 months of imidazole dipeptide formula supplementation containing 500 mg of CRC supplementation (carnosine and anserine, ratio1/3) to 31 healthy participants, the CRC group had not only a better verbal episodic memory performance but also, at functional MRI, a decreased connectivity in the default mode network, the posterior cingulate cortex and the right frontoparietal network, as compared with the placebo group. Furthermore, there was a correlation between the extents of cognitive and neuroimaging changes suggesting that daily CRC supplementation could impact cognitive function and that network connectivity changes may be associated with its effects [28] (Table 2). These findings were confirmed in another RCT including 39 healthy older adults assigned to a CRC supplementation (carnosine and anserine) or placebo for three months. CRC group showed significant preservation in delayed recall verbal memory compared to the placebo group, but not in the immediate recall test, suggesting that CRC supplementation may have a beneficial effect on verbal memory registration, but not on short-term working verbal memory. Blood analysis revealed a decreased secretion of inflammatory cytokines in the CRC group, including CCL-2 (MCP-1) and interleukin (IL)-8. Furthermore, perfusion MRI analysis using arterial spin labeling showed a suppression of the age-related decline in brain blood flow in the posterior cingulate cortex area in the CRC group compared to the placebo group suggesting a protective role of CRC supplementation on brain perfusion [29] (Table 2). Finally, an RCT investigated the effects of L-carnitine supplementation on 50 pre-frail older subjects randomized into two groups (26 in L-carnitine group and 24 in placebo group). Outcome measures included physical frailty status using Fried criteria and Frailty Index (FI) accumulation of deficit, selected frailty biomarkers (IL-6, tumor necrosis factor-alpha, and insulin-like growth factor-1), physical function, cognitive function, nutritional status and biochemical profile. The results indicated that the mean scores of FI and hand grip test were significantly improved in subjects supplemented with L-carnitine as compared to no change in the placebo group. Based on Fried criteria, four subjects (three from the L-carnitine group and one from the control group) transited from pre-frail status to robust after the intervention. The results showed no significant differences after the intervention. There were no significant changes in the blood level of biomarkers and in the secondary outcome variables, i.e., nutritional status, body composition, and cognitive function [30] (Table 2).

Fatty acids

In AD brains, it has been reported the lack of enzyme responsible for converting choline into acetylcholine, therefore, the first dietary lipids proposed as potential therapeutic agents in AD were

lecithin, the major dietary source of choline, and alpha-lipoic acid, both able to increase acetylcholine production [58]. However, results from clinical trials were contrasting and further RCTs are required to evaluate their role as therapeutic supplements in order to delay cognitive impairment. Many epidemiological studies have demonstrated that dietary fatty acids may play a key role in several pathological conditions. Long-chain (LC) PUFAs, such as DHA, EPA, and arachidonic acid (ARA) are among the most studied macronutrients in late-life cognitive disorders and neurodegeneration [59]. In particular, an increasing body of epidemiological evidence suggested that elevated saturated fatty acids (SFAs) could have negative effects on MCI [60], while a clear reduction of risk for cognitive decline has been found in population samples with elevated fish consumption, high intake of monounsaturated fatty acids (MUFAs) and LC PUFAs, particularly n-3 PUFAs [60]. Despite the strong evidence in cognitive decline prevention coming from observational studies, findings coming from RCTs were controversial considering the great heterogeneity of samples and outcome measures as well as neuropsychological tools or MRI findings [31–40] (Table 2). Interesting data have been suggested from one RCT on 65 healthy subjects showing not only a significant increase in executive functions and letter fluency in the n-3-PUFA group compared with placebo, but also neuroimaging modifications after supplementation suggesting a pathobiological effect of n-3 PUFAs [31] (Table 2). In fact, n-3 PUFA supplementation led to significant beneficial effects on white matter microstructural integrity and significant increases in regional gray matter volume compared with placebo in specific regions as left hippocampus, precuneus, superior temporal, inferior parietal and postcentral gyri, and in the right middle temporal gyrus and beneficial effects on carotid intima media thickness and diastolic blood pressure. Improvements in executive functions correlated positively with changes in omega-3-index and peripheral brain-derived neurotrophic factor, and negatively with changes in peripheral fasting insulin [31] (Table 2). In another RCT, n-3 PUFA supplementation was effective on immediate and long-delayed free recall only in healthy lonelier participants. In fact, lonelier people within the placebo condition had poorer verbal episodic memory post-supplementation, as measured by immediate and long-delay free recall, than their less lonely counterparts. This effect was not observed in the n-3 PUFA 1.25 g/day and n-3 PUFA 2.5 g/day supplementation groups. The plasma n-6 PUFAs:n-3 PUFAs ratio data mirrored these findings [32] (Table 2). However, findings from two RCTs showed that oral supplementation with n-3 PUFAs had no statistically significant effect on cognitive functions [33, 34] (Table 2). In particular, in an RCT on 199 older subjects with normal or mild to moderate cognition impairment, low dose n-3 PUFAs (180 mg of DHA+120 mg of EPA) for 6 months had no significant beneficial effects on improvement of cognition or prevention of cognitive decline in older people. However, considering only the cognitively healthy subjects, authors noticed near significant less decrement in global cognitive scores in n-3 PUFA group compared to placebo [33] (Table 2). Moreover, in a large RCT including 2831 older participants, randomized to receive n-3 PUFAs (1 g) and/or lutein (10 mg)/zeaxanthin (2 mg) versus placebo for 5 years no statistically significant differences in change of cognitive scores between groups were reported [34] (Table 2). Furthermore, several RCTs reported promising findings only in specific cognitive domains evaluated with several neuropsychological tests. In fact, in an RCT including 160 healthy participants randomized to multivitamins with fish oil for 16 weeks, the red blood cell n-3/n-6 ratio increases were associated with improvements in spatial working memory [35] (Table 2). Some trials reported promising results in specific cognitive domains with higher doses of LC PUFAs compared to general dietary intake levels. Interestingly, an RCT suggested a potential role in improving cognitive function of LC PUFAs also at low doses of supplementation similar to general dietary intake. In fact, in 113 nondemented older Japanese participants, after 4 weeks of supplementation with LC PUFA-containing oil (DHA 300 mg/day, EPA 100 mg/day, and ARA 120 mg/day) or purified olive oil as placebo, changes in P300 latency, a measure of cognitive processes, were significantly different between the placebo group and the LC PUFA group. Significant increases in DHA and ARA contents in plasma phospholipids were observed in the LC PUFA group, while no changes were observed in the placebo group [36] (Table 2). In another RCT conducted on 44 cognitively healthy individuals, the recall of object locations was significantly better after n-3 PUFA supplementation (daily dose of 1.320 mg EPA+880 mg DHA for 26 weeks) compared with placebo. No significant correlation between changes in memory performance and omega-3-index were observed, suggesting that memory benefits were not associated in a simple linear fashion with changes in omega-

3-index [37] (Table 2). Furthermore, in 86 healthy older adults aged 50–70 years who reported subjective memory deficits, a RCT investigating six months of supplementation with a DHA-rich fish oil or a multinutrient dietary supplement containing a number of potentially cognitive enhancing components including DHA, phosphatidylserine, vitamin B12, folic acid, and Ginkgo biloba on cerebral hemodynamics showed no effect of both the active treatments on either the near infrared spectroscopy (NIRS) or cognitive outcomes. Furthermore, a doubling in concentration of DHA and around a 50% increase in EPA following both active treatments suggesting that adherence to the treatment was very good [38] (Table 2). In another recent RCT, 92 cognitively healthy subjects with coronary artery disease aged 45 to 80 years were randomized to receive either 1.9-g/day n-3 PUFA treatment or placebo for 12 weeks. In this trial, n-3 PUFA treatment did not reduce depressive symptom severity compared with placebo, despite n-3 PUFA treatment significantly increased plasma EPA and DHA concentrations. Treatment did not improve cognitive performance; however, n-3 PUFAs significantly increased verbal memory compared with placebo in a subgroup of nondepressed patients [39] (Table 2). Finally, in a small RCT on 21 cognitively healthy older adults (62–80 years) with subjective memory impairment, dietary fish oil supplementation (EPA+DHA, 2.4 g/day) increased red blood cell DHA and EPA content, working memory performance, and BOLD signal by functional MRI in the posterior cingulate cortex during greater working memory load suggesting enhanced neuronal response to working memory challenge [40] (Table 2).

Summary of evidence

Among 15 selected RCTs published in the last four years that evaluated the efficacy of nutritional intervention through supplementation of dietary macronutrients in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years [26–40], there were 5 RCTs investigating protein supplementation [26–30] and 10 RCTs investigating fatty acid supplementation [31–40]. Of these trials, for protein supplementation, 4 RCTs suggested an impact of this approach in improving specific cognitive domains [26–29], and in one RCT, L-carnitine supplementation had a favorable effect on the functional status and fatigue in prefrail older adults, without effect on cognitive function [30]. For fatty acid supplementation, mainly LC PUFAs, 5 RCTs suggested an impact of this approach in improving specific cognitive domains, MRI findings, and/or cognitive-related biomarkers [31, 35–37, 40], 2 RCTs showed an impact of LC PUFA supplementation only in selected subgroups [32, 39], and 3 RCTs did not find significant improvement in cognitive functions, MRI findings, or cognitive-related biomarkers after LC PUFA supplementation [33, 34, 38].

Micronutrients

Non-flavonoid polyphenols

Table 3 shows selected RCTs published in the last four years (2014–2017) that evaluated the efficacy of nutritional intervention through supplementation of dietary micronutrients in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years [41–52]. Several classes of polyphenols have been investigated for their potential anti-aging and neuroprotective properties, including flavonoids, commonly found in berries, grapes and red wine, and non-flavonoids, i.e., curcumin from turmeric and resveratrol from grapes and red wine [61]. An increasing body of evidence suggested that consumption of polyphenols such as resveratrol and flavonoids may have potential beneficial effects on cognition, particularly on declarative and spatial memory, mainly in cognitively healthy individuals [61]. However, results from RCTs were contrasting considering also the methodological inconsistencies of studies. On the other hand, findings from observational studies suggested that moderate consumption of red wine, rich in specific polyphenolic compounds such as quercetin, myricetin, catechins, tannins, anthocyanidins, resveratrol, and ferulic acid, has been associated with a lower incidence of cognitive decline, suggesting a protective role against dementia [62]. These data were confirmed from recent RCTs [41, 42] (Table 3). In fact, a trial including 46 cognitively healthy older adults randomly assigned to receive a daily intake of 200 mg of resveratrol and 320 mg of quercetin or placebo showed that supplementary resveratrol over a period of 26 weeks improved retention of words over a 30 min delay and functional connectivity of the hippocampus with frontal, parietal, and occipital areas in healthy older overweight adults compared with placebo [41] (Table 3). Furthermore, a RCT involving 80 postmenopausal women aged 45–85 years and randomly assigned to receive a daily intake of resveratrol (75 mg twice daily) or placebo for 14 weeks showed

that, compared to placebo, resveratrol elicited 17% increases in cerebrovascular responsiveness (CVR) to both hypercapnic and cognitive stimuli. Significant improvements were observed also in the performance of cognitive tasks in the domain of verbal memory and overall cognitive performance, which correlated with the increase in CVR [42] (Table 3). Mood tended to improve in multiple measures, although not significantly. These findings indicated that regular consumption of a modest dose of resveratrol can enhance both cerebrovascular function and cognition in post-menopausal women, potentially reducing their heightened risk of accelerated cognitive decline [42] (Table 3). Among non-flavonoid polyphenols, curcumin has been extensively reported to demonstrate many beneficial biological effects including anti-cancer, antioxidant and anti-inflammatory activities [63].

For the prevention of cognitive-related outcomes in older age, promising results were reported in a one-year RCT in 96 cognitively normal subjects randomized to receive placebo or 1500 mg/d BiocurcumaxTM. A significant time × treatment group interaction was observed for global cognitive function, explained by a function decline in the placebo group at 6 months that was not found in the intervention group [43] (Table 3).

Flavonoids

Flavonoids [flavanols (catechin, epicatechin, epigallocathechin, and epigallocatechingallate-EGCG), flavonols (quercetin and kaempferol), flavones (luteolin and apigenin), isoflavones (daidzein and genistein), flavanones (esperetin and naringenin), and anthocyanidins (pelargonidin, cyanidine, and malvidin) have also been proposed to prevent or treat cognitive impairment or dementia [56, 64]. Recent RCTs showed significant improvements in some cognitive domains after flavonoid interventions [65]. However, the great heterogeneity in sample, flavonoid dose, follow-up and cognitive tests used led to inconsistent findings [65]. In a very interesting RCT on 37 healthy older adults who consumed a high cocoa flavanol-containing diet (900 mg cocoa flavanols and 138 mg of epicatechin) or a low-dose one (10 mg cocoa flavanols and < 2 mg epicatechin) with or without aerobic exercise for 12 weeks, the high-flavanol intervention was found to enhance dentate gyrus (DG) function measured by functional MRI and by cognitive testing, suggesting the crucial role of DG dysfunction in age-related cognitive decline and the potential beneficial effects of flavonoid supplementation on DG function [44] (Table 3). On the contrary, in a trial including 300 cognitively healthy postmenopausal women randomized to receive 25 grams of isoflavone-rich soy protein for 2.7 years, long-term changes in isoflavonoids were not associated with global cognition and episodic memory, although greater isoflavonoid exposure was associated with decrements in general intelligence [45] (Table 3). Promising results come from other two trials with an 8-week follow-up [46, 47] (Table 3). In particular, in a RCT including 37 healthy participants randomized to receive two different flavanone-rich supplementations, high flavanone and low flavanone orange juice drinks, global performance, executive function, and episodic memory, and immediate recall were significantly better after the high flavanone drink than the low flavanone drink [46] (Table 3). Similar positive findings were found in the second RCT for a drink containing a high dose of cocoa flavanols (993 mg/day) compared to a low dose drink (993 mg/day) in cognitively healthy participants for specific cognitive domains (i.e., executive function and verbal fluency) suggesting a possible protective role in age-related cognitive dysfunction, possibly through an improvement in insulin sensitivity [47] (Table 3). Finally, in a 5-week RCT, an intervention with a berry beverage based on a mixture of Swedish berries known to be rich in polyphenols or carotenoids (lycopene) was compared with the effects of a control beverage matched with respect to monosaccharide content and distribution, pH, and volume. The berry beverage resulted in a modest (∼5%) but significant improvement in working memory in comparison with the control beverage [48] (Table 3). Furthermore, the berry beverage significantly reduced the concentrations of total and low density lipoprotein (LDL) cholesterol. On the contrary, the control beverage resulted in significantly increased fasting glucose concentrations from baseline [48] (Table 3).

Homocysteine-related and antioxidant vitamins

A possible modifiable risk factor of dementia is an elevated plasma Hcy level. In fact, Hcy may be toxic for neurons and vascular endothelial cells [66], and cross-sectional and prospective studies have shown associations between elevated Hcy levels and cognitive decline and dementia [67]. Hcy levels can be lowered by supplementation with folic acid (vitamin B9) and vitamin B12 [68]. Although observational studies have shown a strong association between poor vitamin B6, B12, and folate levels and increased risk

of dementia, suggesting a preventive and protective role of these micronutrients, evidence from RCTs appeared to be unclear [49–52] (Table 3). In fact, in two RCTs, no significant effect of supplementation of Hcy-related vitamins on cognitive function were found [49, 50] (Table 3). In particular, in a large RCT on 2.919 older participants with elevated Hcy levels, a 2-year folic acid and vitamin B12 supplementation did not significantly improve cognitive performance in all four cognitive domains investigated (episodic memory, attention and working memory, information processing speed, and executive function). Interestingly it was reported a small difference in global cognition, that the authors concluded as attributable to chance [49] (Table 3). The other RCT included 201 healthy cognitive older adults with moderate vitamin B12 deficiency. In this one-year follow-up trial, there was no effect of B12 supplementation peripheral nerve or central motor function outcome or cognitive function [50] (Table 3). However, another RCT suggested more promising findings with a supplementation containing 800 μg/day of folate, 10 mg of vitamin B6, and 25 μg of vitamin B12 in 83 older patients with hyperhomocysteinemia. This supplementation improved cognitive function in terms of global cognitive scores and four subtests (mental speed, visuo-spatial ability, working memory, and visual memory) [51] (Table 3). Finally, among RCTs using as supplementation antioxidant vitamins (i.e., vitamins A, C, and E), the Prevention of Alzheimer's Disease by Vitamin E and Selenium Trial (PREADVISE) was a double-blind RCT conducted as an ancillary study to a cancer prevention trial (SELECT), both of which evolved into observational cohort studies. This trial investigated whether the supplements vitamin E and selenium used alone or in combination would prevent new AD or dementia cases [52] (Table 3). The results of the PREADVISE showed that neither vitamin E or selenium (with 5.4 ± 1.2 years of supplement use) had a significant preventive effect on incidence of dementia. One possible explanation for the negative findings is that the trial met only 75% of its planned accrual. Nevertheless, although largely negative, this was the first, large-scale primary prevention trial to investigate the effect of antioxidant supplements on reducing dementia incidence [52] (Table 3).

Summary of evidence

Among 12 selected RCTs published in the last four years that evaluated the efficacy of nutritional intervention through supplementation of dietary micronutrients in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years [41–52], there were 3 RCTs investigating non-flavonoid polyphenol supplementation [41–43], 5 RCTs investigating flavonoid supplementation [44–48], and 4 RCTs investigating intervention with homocysteine-related and antioxidant vitamins [49–52]. Of these trials, for non-flavonoid polyphenol supplementation, all the reviewed RCTs suggested an impact of this approach in improving specific cognitive domains and/or MRI findings [41–43]. For flavonoid supplementation, while 4 RCTs suggested an impact of this approach in improving specific cognitive domains and/or MRI findings [44, 46–48], in one RCT, long-term changes in isoflavonoids were not associated with global cognition and increasing isoflavonoid exposure was associated with decrements in general intelligence but not memory [45]. Finally, for the intervention with homocysteine-related and antioxidant vitamins, only an RCT suggested an impact of vitamin B supplementation in improving specific cognitive domains [51], while the other 3 reviewed RCTs did not find significant improvement in cognitive functions, dementia incidence, or cognitive-related biomarkers after homocysteine-related or antioxidant vitamin supplementation [49, 50, 52].

RISK OF BIAS AND OVERALL QUALITY OF EVIDENCE

Examining all the 35 included RCTs in the present systematic review [17–52] (Tables 1–3), bias was detected predominantly in the domains of random sequence generation (selection bias) (12/35 studies, 34% of RCTs with unclear risk of bias) and blinding of outcome assessment (detection bias) (6/35 studies, 17% of RCTs with unclear risk of bias). Using the GRADE approach, the overall quality of evidence was judged as moderate.

POSSIBLE NEUROBIOLOGICAL MECHANISMS UNDERLYING NUTRITIONAL PREVENTION AND COGNITIVE-RELATED OUTCOMES IN COGNITIVELY HEALTHY OLDER ADULTS

Various theories have led to the evaluation of nutritional factors as potential modifiers of the risk of cognitive impairment in older age [57]. Oxidative

stress has long been considered to play a major role in cognitive decline and neurodegenerative disorders, considering its involvement in cell death, membranes peroxidation and Aβ deposition [69]. Thus, it is plausible that, by counteracting oxidative stress, antioxidant-rich foods might afford protection from neurodegenerative diseases. In this regard, the MeDi is a plant-based, antioxidant-rich dietary pattern reputed for its many health benefits [70]. Therefore, individuals who adhere to a MeDi (low intake of meat and dairy, high intake of fruit, vegetables, and fish) have fewer vascular risk factors and reduced plasma glucose and serum insulin concentrations, insulin resistance, and markers of oxidative stress and inflammation [71]. Consequently, in the older population, a MeDi supplemented with EVOO or nuts may counteract age-related cognitive decline [72]. The beneficial effect of MeDi on cognition probably stems from the abundance of not only antioxidants but also anti-inflammatory agents that this diet may provide. The supplemental foods, EVOO, and nuts, are particularly rich in phenolic compounds that might counteract oxidative processes in the brain, leading to neurodegeneration [72, 73]. Polyphenols can ameliorate neurological health by additional mechanisms, including improved cerebrovascular blood flow, modulation of neuronal signaling, enhanced synthesis of neurotrophic factors, and stimulation of neurogenesis [74]. The antiaging effects of polyphenols could be due to several related mechanisms, among which are the prevention of oxidative stress, sirtuin 1 activation and inflammaging modulation, via regulation of some signaling pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [75]. On the other hand, nuts, particularly walnuts, contain sizeable amounts of α-linolenic acid, the vegetable n-3 PUFA [60]. Administration of α-linolenic acid has been found to enhance brain plasticity and exert an antidepressant effect in experimental animals [76]. The fish oil and EVOO contain a lot of n-3 PUFAs, part of the neural membrane functioning, increase the activity of antioxidation enzymes, protecting against oxidative stress, neuronal death, and the formation and aggregation of Aβ in the brain [77]. Moreover, fatty acid intake might affect the development of cognitive impairment by way of the influence of fatty acids on atherosclerosis and thrombosis [78]. Fatty acids, especially those in ester phospholipids, control the structure and function of biological membranes, including membranes in nervous tissues and erythrocytes [79, 80]. Thus, fatty acids strongly influence membrane fluidity. The central nervous system has the second highest concentration of lipids after adipose tissue. The brain lipids contain very high amounts of LC PUFAs, particularly ARA and DHA. These 2 LC PUFAs, which are the major constituents of neural cell membrane phospholipids, belong to the n-6 and n-3 PUFA families and can only be obtained from the diet [79]. Supplementation with n-3 PUFAs, which have anti-inflammatory effects, might protect against cognitive decline and AD: results from some observational studies [79, 81, 82] examining the relationship between PUFAs and cognitive decline or incident dementia are encouraging, but those from RCTs are conflicting.

Among micronutrients, curcumin has been reported to have a wide variety of effects, including decreasing Aβ plaques and microglia formation, delaying neurons degradation, anti-inflammatory and antioxidant activities [83]. In addition, in *in vitro* studies, grape seed polyphenolic extracts have been reported to be involved in modulation of tau-mediated neuropathological mechanisms [84]. For Hcy-related vitamins, folate and B12 vitamin have a crucial role in the formation of methionine and S-adenosyl methionine, a common methyl donor involved in generation of neurotransmitters, phospholipids, and myelin [85]. Furthermore, folic acid (vitamin B9), vitamin B6, and vitamin B12 are the most effective agents able in reducing serum Hcy levels and this is another important protective activity to underline. In fact, is well known that hyperhomocysteinemia is an important risk factor for cognitive decline and dementia [66, 67]. The underlined mechanisms include also nucleic acids and neurotransmitters hypomethylation, oxidative stress, Aβ production and overstimulation of N-methyl-D-aspartate receptors resulting in neurotoxicity and apoptosis [86]. Therefore, deficiency of folate and B12 and B6 vitamins have several neuropathological effects [87, 88]. Interestingly the integration of B12 vitamin and folic acid is not only useful for cognitive decline prevention but also in protection from neuropathy, depression, and cerebrovascular diseases [89]. Vitamin B6 supplementation has been studied to ameliorate depression symptoms, learning disability, and memory decline as result of its effects on synapses in the hippocampus [87]. Finally, it is easily understandable the potential preventive role of micronutrients known to have antioxidant properties such as vitamins A, C, and E [90]. In particular, vitamin E has been reported to scavenge oxygen free radicals, maintain the integrity and

stability of membranes and reduce Aβ toxicity, as result of the inhibition of peptide deposition and its induced clearance [91]. Vitamin C, based on its crucial role as extracellular antioxidant, is the only factor able to prevent lipid peroxidation caused by water-soluble free radicals. In addition, it has been found to cooperate with vitamin E, restoring the impaired activity of the oxidized form [92].

DISCUSSION

In the last decade, while the association between diet and cognitive function or dementia has been largely investigated in observational studies, there was a lack of evidence from RCTs dealing with the prevention of late-life cognitive disorders though dietary intervention in older adults without cognitive dysfunction In the present article, we systematically reviewed RCTs published in the last four years (2014–2017) exploring nutritional intervention efficacy in preventing the onset of late-life cognitive disorders and dementia in cognitively healthy subjects aged over 60 years and using different levels of investigation (i.e., dietary pattern changes/medical food/nutraceutical supplementation/multidomain approach and dietary macro- and micronutrient approaches). From the reviewed RCTs, there was moderate evidence that nutritional intervention through dietary pattern changes, medical food/nutraceutical supplementation, and multidomain approach improved specific cognitive domains or cognitive-related blood biomarkers. Furthermore, there was convincing evidence that protein supplementation improved specific cognitive domains or functional status in prefrail older adults without effect on cognitive function. For fatty acid supplementation, mainly LC PUFAs, there was emerging evidence suggesting an impact of this approach in improving specific cognitive domains, MRI findings, and/or cognitive-related biomarkers also in selected subgroups of older subjects although some results were conflicting. Among selected RCTs that evaluated the efficacy of nutritional intervention through supplementation of dietary micronutrients, there was evidence of an impact of non-flavonoid polyphenol and flavonoid supplementations in improving specific cognitive domains and/or MRI findings. Finally, there was only low evidence suggesting efficacy of intervention with homocysteine-related and antioxidant vitamins in improving cognitive functions, dementia incidence, or cognitive-related biomarkers in cognitively healthy older subjects.

In the last four years, several meta-analyses and systematic/scoping reviews investigated the efficacy of different nutritional supplementations in preventing late-life cognitive disorders in cognitively healthy older adults [78, 93–95]. However, these meta-analyses and systematic/scoping reviews investigated also observational studies and not only RCTs [78, 95], included also younger subjects [93], and were limited to specific macronutrients (i.e., n-3 PUFAs) [78, 93–95], micronutrients [93, 94], or dietary pattern changes/nutraceuticals [78, 95]. In particular, some of these studies found that n-3 PUFAs were associated with better global cognition and some specific cognitive domains [78, 94, 95], B vitamins, and vitamin E supplementations did not affect cognition [93] or had limited efficacy [94, 95], while adherence to the MeDi was significantly associated with better cognitive performance and less cognitive decline [78].

The absence of disease-modifying treatment for AD patients leads to the investigation of multimodal alternative therapeutic or preventive approaches by targeting modifiable risk factors. Therefore, in the last years, a growing interest has concerned the relation between nutrients and cognitive impairment in the earlier phases, considering the multifactorial effects of nutrition in human diseases. In fact, it is well known that dietary habits may influence several cardiometabolic risk factors, as visceral adiposity, blood pressure, glucose-insulin metabolism, lipids levels, but also hepatic function, endothelial health, microbiome function, and several biological processes as oxidative stress, inflammation, both involved in human aging. Despite several promising findings coming from observational studies [4], evidence suggesting a potential preventive effectiveness of nutritional intervention in healthy elderly to delay the onset of cognitive decline are still scarce and quite contrasting. Considering that is unlikely that a single nutrient could significantly improve cognition and delay cognitive impairment, several observational studies and RCTs proposed combination of micro/macronutrients or medical foods/nutraceuticals as potential preventive approaches in elderly with promising results [4, 78, 94, 95]. Furthermore, a multidimensional approach consisting in healthy lifestyle (healthy dietary habits in combination with physical activity) seems the best intervention in the elderly. In fact, it is well known that there is a strong bidirectional interaction between cognitive performance

and other main outcomes in the eldery that have to be considered as physical and cognitive frailty and disability [96].

However, some limitations should be reported for the present systematic review article. An important limitation was linked to the great heterogeneity of included RCTs not only in terms of study samples and trial durations, but also in relation to the outcome measures and nutrients intake quantification. This heterogeneity made really difficult to give clear answers about the efficacy of dietary intervention in older adults without cognitive dysfunction. However, there are several interesting concepts coming from the reviewed RCTs to underline. The first one was the emerging use of innovative measures of dietary habits, not only daily questionnaire but also biomarkers dosage as blood exams or urinary excretion. This resulted into an objective quantification of nutrient supplementation but also of nutritional status of patients at baseline. Furthermore, as shown in the present systematic review, recent RCTs underlined the importance to consider emerging cognitive-related outcomes in order to achieve more significant and objective results. Therefore, in addition to clinical scales and cognitive tests, serum and cerebrospinal fluid biomarkers, neuroimaging and other cognitive-related biomarkers have been proposed. As a result, these findings could give us the possibility to better understand and quantify the nutrition-related impact on cognitive impairment and AD pathobiology. In conclusion, dietary pattern change/multidomain approaches, macronutrient (i.e., proteins and LC PUFAs) and micronutrient (i.e., non-flavonoid polyphenols and flavonoids) supplementations could be really effective in achieving cognitive-related outcomes in healthy older subjects without cognitive dysfunction. However, to obtain more statistically significant and reliable results, RCTs would be conducted in larger selected samples characterized by well-defined cognitive function status, nutritional and dietary habits at baseline, with longer follow-up, and would include further objective measures of cognitive-related outcomes as blood or cerebrospinal fluid biomarkers and neuroimaging findings. Some of these RCTs are currently ongoing investigating the efficacy of a MeDi pattern (MedLey study) [97], a protein enriched diet with lean red meat combined with a multi-modal exercise program [98], and a MeDi plus aerobic exercise [Lifestyle Intervention in Independent Living Aged Care (LIILAC) study] [99] on cognitive function and psychological wellbeing in cognitively healthy older adults.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9940).

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The Relationship between Obstructive Sleep Apnea and Alzheimer's Disease

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Abstract. Obstructive sleep apnea (OSA) and Alzheimer's disease (AD) are highly prevalent conditions with growing impact on our aging society. While the causes of OSA are now better characterized, the mechanisms underlying AD are still largely unknown, challenging the development of effective treatments. Cognitive impairment, especially affecting attention and executive functions, is a recognized clinical consequence of OSA. A deeper contribution of OSA to AD pathogenesis is now gaining support from several lines of research. OSA is intrinsically associated with disruptions of sleep architecture, intermittent hypoxia and oxidative stress, intrathoracic and hemodynamic changes as well as cardiovascular comorbidities. All of these could increase the risk for AD, rendering OSA as a potential modifiable target for AD prevention. Evidence supporting the relevance of each of these mechanisms for AD risk, as well as a possible effect of AD in OSA expression, will be explored in this review.

Keywords: AD risk, Alzheimer's disease, amyloid, obstructive sleep apnea, OSA phenotypes

INTRODUCTION

Obstructive sleep apnea (OSA) is a common medical condition with increasingly recognized impact on global health worldwide. Obstructive apneic events occur when there is transient partial or complete closure of the upper airway during sleep [1]. These apneic episodes are associated with cycles of hypoxia/hypercapnia/reoxygenation, transitory increases in intrathoracic pressure, hemodynamic disruptions, and recurrent brain arousals with sleep fragmentation [2]. OSA is the most common form of sleep-disordered breathing (SDB) accounting for about 85% of the cases, with central sleep apnea being less common [3]. OSA is frequently classified for both clinical and research purposes according to the Apnea-Hypopnea Index, AHI (number of apneas and hypopneas per hour of sleep). While apneas have been consistently defined as decreases in respiratory airflow greater than 90% for more than 10 seconds, one conundrum in the field is that there are at least two commonly used definitions of hypopneas. The most recent revision by the American Academy of Sleep Medicine (AASM) defines hypopneas as decreases in inspiratory airflow of more than 30% for >10 seconds, associated with a drop of at least 3% in oxygen saturation or

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arousal (AHI3a) [4]. The older definition of hypopneas, which was used in research for many years, required an oxygen desaturation of at least 4%, irrespective of whether an arousal occurred, and indices using this criterion are sometimes denoted AHI4%. OSA severity has traditionally been predicated on AHI4% values in which 5–14 events/hour constitutes mild OSA, 15–29 events/hour constitutes moderate OSA, and ≥30 events/hour constitutes severe OSA. The fact that these same cut-offs are inappropriately applied to AHI3a may account for some of the disparate results in the sleep research literature. Some use the term OSA syndrome (OSAS) to refer to the presence of OSA plus daytime sleepiness.

Clinically, OSA can remain asymptomatic, accounting for its presumed high underdiagnosis rate, or present with a wide variety of symptoms. These can range from mild snoring and feelings of unrefreshing sleep, to several degrees of excessive daytime sleepiness (EDS) [5], cognitive impairment (especially affecting attention and executive functions) [6], depression, and functional impairment [7]. OSA not only impacts quality of life, but is also associated with increased risk of work and traffic accidents [8, 9], adding to its importance as a major health concern that should be effectively recognized and treated.

OSA is also often accompanied by several comorbidities. All aspects of the metabolic syndrome, namely insulin resistance or diabetes [10], dyslipidemia [11], hypertension [12, 13], and obesity [14], have been associated with OSA. It has been suggested that the metabolic syndrome or "syndrome X" should also comprise OSA and be then called syndrome "Z". Cardiac arrhythmias, heart failure, and stroke are also documented more frequently among OSA patients [15–18]. Besides its recognized direct effect on cognitive performance, gathering evidence is now supporting a role of OSA in dementias' pathophysiology.

Alzheimer's disease (AD) is the most common form of dementia worldwide, accounting for more than 70% of all cases. Vascular dementia and other neurodegenerative types of dementia account for most of the remaining cases. More than 4.7 million people aged over 65 years in the United States are now affected by AD, and its prevalence is expected to increase up to 13.8 million people in 2050 if new preventive and treatment measures are not implemented [19]. Its main neuropathological hallmarks, extracellular amyloid-β (Aβ) plaques and intraneuronal neurofibrillary tangles (NFT), characteristically accumulate throughout the brain, culminating in the progressive and irreversible cognitive decline seen in AD patients [20, 21]. A combination of genetic and environmental factors is now considered an accepted framework to explain individual predisposition for AD development; however, its specific underlying pathophysiological mechanisms are still elusive. Age and genetic background, including the presence of the ApoE4 genotype, are important non-modifiable risk factors for AD. Cognitive reserve and physical activity are recognized protective factors and numerous medical diseases such as traumatic brain injury, depression, midlife obesity, diabetes, and cardiovascular and cerebrovascular disease, have all been associated with increased risk of AD [22].

OSA, besides being more prevalent in older populations (as is AD) [23], has also been associated with both cognitive decline [24] and dementia [25]. Several mechanisms that characterize OSA, such as disruption of sleep architecture, intermittent hypoxia, increased oxidative stress, intrathoracic pressure changes, and cardiovascular comorbidities, could contribute to an increased risk of AD. Exploring the evidence supporting these possible interactions will be the focus of this review. The possible effects of AD on OSA expression will also be briefly mentioned.

EVIDENCE OF A LINK BETWEEN OSA AND AD

Evidence from animal, epidemiological, and human AD studies suggests an interdependent relationship between OSA and AD. These are both highly prevalent diseases in older populations and frequently coexist. A recent meta-analysis found that AD patients have a 5-fold increased risk of presenting with OSA compared to age-matched controls, and that about 50% of AD patients experience OSA after their initial diagnosis [26].

Conversely, OSA may promote the worsening of existing AD. For example, in triple transgenic AD mice, induced chronic intermittent hypoxia was associated with increased levels of brain $A\beta_{42}$ [27] and an increase of tau phosphorylation [28] compared to control mice. In humans, earlier studies from Ancoli-Israel et al. showed a strong correlation between severity of OSA and severity of AD symptoms [29], suggesting that AD clinical expression is aggravated by OSA in patients with full-blown dementia.

The interaction between these two diseases could even begin before overt clinical symptoms are present in AD, and several studies support this hypothesis. First, in a prospectively longitudinal study, 105 elderly women with OSA had a higher risk of developing mild cognitive impairment (MCI) or dementia compared to 193 women without OSA (adjusted OR, 1.85; 95% CI, 1.11–3.08) [30]. Second, our group documented a positive association between the presence of reported OSA and an earlier age of MCI onset, as well as a possible delay of this effect in continuous positive airway pressure (CPAP) treated subjects [25]. In addition, our recent meta-analysis determined a 1.55, 1.65, and 3.78 increased risk of AD, cognitive impairment, and preclinical AD, respectively, in patients with sleep problems compared to controls. Sub-group analyses also revealed that OSA participants had approximately twice the risk compared to non-OSA participants of cognitive decline and/or AD [31].

Studies evaluating AD specific cerebrospinal fluid (CSF) biomarkers further support this hypothesis. In a recent 2-year follow up study, baseline OSA severity was associated with higher rate of CSF $A\beta_{42}$ decline and with a trend toward increased cortical Pittsburgh compound B (PiB)-PET uptake [32] in cognitively normal elderly. In another study, among subjects with subjective cognitive impairment, the ones with untreated OSA had higher T-tau/$A\beta_{42}$ ratio and lower levels of $A\beta_{42}$ compared to CPAP treated and non-OSA subjects [33].

Clinical trials exploring the effect of CPAP treatment on cognition and AD also strengthen the suspected link between OSA and AD. A large randomized controlled trial (RCT) demonstrated a mild but measurable improvement of executive function in OSA patients treated for 6 months with CPAP versus untreated subjects [34]. In mild to moderate AD subjects with OSA, a small RCT showed that CPAP treatment partially improved verbal learning, memory, and executive functions [35]. A later reassessment of part of these subjects suggested that sustained use of CPAP improved sleep and mood, and slowed cognitive decline [36]. This initial finding was corroborated by a 3-year pilot study performed in France where AD patients that underwent CPAP treatment showed significantly slower cognitive decline when compared to the non-CPAP AD group [37].

In summary, growing evidence from animal and human studies supports an interdependent relationship between OSA and AD. The immediate deleterious effect of OSA in cognition, especially on executive function and attention, may contribute to a worsening of the AD clinical presentation, and in addition, OSA may influence relevant ADs pathophysiological mechanisms in preclinical AD stages before overt cognitive symptoms exist. Importantly, adequate diagnosis and treatment of OSA may hold a promising beneficial preventive effect in preclinical AD as well as in slowing cognitive decline in clinical AD.

OSA PHENOTYPES

OSA has been extensively studied in middle-aged adults, where its underlying anatomical causes and associated comorbidities are well characterized. Recent studies have focused on OSA in older populations, and the existence of two separate entities is now debated. The terms "age-dependent", in which aging determines pathogenesis, and "age-related", where pathogenesis occurs during a specific age range, have been proposed to define old and middle-age OSA, respectively [38, 39]. Multiple lines of evidence support this categorization. First, epidemiological studies show a prevalence of OSAS in middle-aged populations different from the estimated in the elderly [39–43]. A recent large prospective study assessing AHI3a by polysomnography (PSG) determined a prevalence of mild to moderate OSA of 83.8% in men and 60.8% in women, while severe forms were noted in 49.7% and 23.4% of men and women respectively. Older age (>60 years) was associated with significantly higher prevalence of moderate to severe OSA and attenuation of the sex discrepancy compared to younger subjects [44]. Age-dependent structural and functional changes of the upper airways could account at least partially for these differences [45]. In fact, higher airway resistance [46], decreased pharyngeal diameter [47, 48], increased pharyngeal fat deposits [50], and sleep-induced changes in the upper airway muscular activity [49], were all found more frequently in the elderly compared to younger subjects, although other studies showed contradictory results [50–53]. Alternatively, sleep-architecture modifications that occur with aging, as sleep fragmentation, reductions of slow wave sleep (SWS) duration [54], and increased percentage of non-rapid eye movement (NREM) stages 1 and 2, could also determine an increased susceptibility to OSA [38]. Possibly, all of these changes could add to, or accentuate, preexisting middle-age OSA [41].

OSA also often presents differently in these two age groups. For example, contrasting with the higher prevalence of OSA, snoring has been found to be less frequent in older populations [55]. Furthermore, symptoms such as EDS, snoring, nocturia, and mild cognitive complaints, that are viewed as pathological in middle-aged adults and should prompt OSA evaluation, may be neglected and considered part of "normal aging" in older adults. OSA in the elderly may also be masked by a more heterogeneous presentation mixed with other health problems, which may obscure the diagnosis [41].

Epidemiological studies on OSA mortality have shown conflicting results. While early reports pointed to higher mortality rates in older OSA patients [56, 57], in other studies, OSA has been linked with increased mortality only if severe or in patients younger than 50 [58]. Recent results from longitudinal cohorts that included older subjects (>65), have shown an increased mortality in older OSA patients only when associated with EDS [59], and although in 40–70 year-olds it determined increased mortality, this association was not found in those 70 and older [60]. In other studies mortality rates in elderly OSA populations are found to resemble those of younger subjects without OSA [61–63]. This has been hypothesized either to relate to a preconditioning cardiovascular protective effect of chronic exposure to intermittent hypoxia in older adults with OSA [64], to a greater tendency for fatal cardiovascular outcomes in younger OSA patients [45], or to survivor bias. Some studies, but not all, suggest that elderly may be less susceptible to OSA related cardiovascular (but not brain) morbidity [55, 63, 65]. Furthermore, obesity, while frequent and relevant to mortality in middle-age OSA, may not be present and even be associated with better outcomes in older subjects [66]. A more consistent view prevails on the beneficial effect on quality of life and morbidity/mortality for both younger and older populations with CPAP treatment [45, 67, 68].

In conclusion, the existence of two separate OSA clinical phenotypes is still a matter of debate. While the clinical manifestations and associated morbidities may be somewhat different in these age groups, it seems reasonable to argue that part of the increased prevalence still derives from the aging of middle-age OSA patients. We believe in a contribution of both middle-age and old-age predisposing factors, acting with different weight in each phase of the continuum of chronological age (Fig. 1).

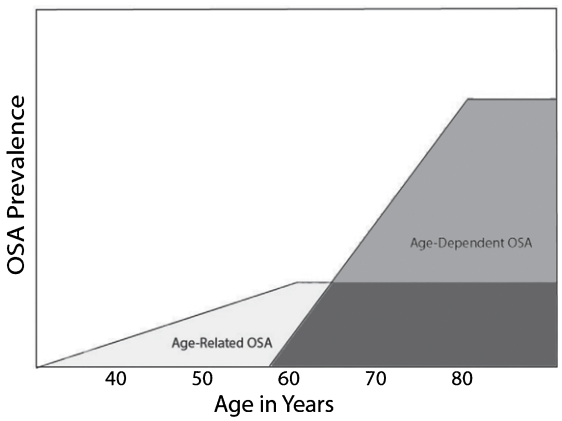

Fig. 1. Proposed prevalence of OSA age-phenotypes. Age-Related OSA would be more common in younger subjects, with its prevalence stabilizing in older age. Age-Dependent OSA prevalence would start to increase in older ages, contributing to the higher prevalence of OSA in this age group.

THE POSSIBLE LINKS BETWEEN OSA AND AD

Effects of sleep disturbances

OSA causes sleep fragmentation

The interplay between sleep and cognition has been vastly explored and its influence on attention, executive function, and memory consolidation is well recognized (for reviews on this topic, see [69, 70]). Experimental studies with rodents have documented that sleep is important for hippocampal neurogenesis [71] and synaptic plasticity [72], and that sleep fragmentation is associated with decreased hippocampal plasticity and spatial learning [73, 74]. OSA fragments sleep architecture due to recurrent brain arousals resulting from reflex responses initiated by upper airway mechanoreceptors and central and peripheral chemoreceptors. This may have not only a direct impact on cognitive performance by disrupting sleep-related memory and attention promoting processes, but also potentially by increasing the risk for dementia.

Two large cross-sectional studies have shown an association between poor sleep quality and worse cognitive outcomes in older populations [75, 76]. In a study performed in cognitively normal individuals, reduced sleep efficiency correlated with lower CSF $A\beta_{42}$ levels, assumed to correspond to preclinical AD [77]. In another study, poor sleep quality reported by healthy adults at increased risk for AD, was associated with CSF biomarker patterns of AD

[78]. Recently, a large prospective study established a robust association specifically between sleep fragmentation and both increased incidence of AD and rate of cognitive decline [79]. At a mean follow-up of 3-years, subjects with higher sleep fragmentation levels had a 1.5-fold increased risk to develop AD compared to subjects with low sleep fragmentation, evaluated by actigraphy.

In parallel, sleep has been suggested to be a fundamental player in brain toxic metabolite clearance processes [80]. Recently, circadian fluctuations of Aβ CSF levels were described, with characteristic increases in wakefulness and decreases during sleep, suggesting that sleep decreases Aβ production and promotes Aβ clearance [81]. Adding to this, chronic sleep disruption was associated with increased Aβ plaque deposition in amyloid-β precursor protein (AβPP) transgenic mice [81]. Finally, Lucey et al. recently compared CSF Aβ kinetics in sleep deprived subjects compared to normal sleeping controls, finding a 25–30% increase in overnight soluble $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ in the former group, suggesting that sleep deprivation contributes to AD risk by promoting Aβ production [82]. In conclusion, sleep appears to play a key role in the production-clearance dynamics of Aβ, which if disturbed could predispose to AD pathogenesis [77, 81]. This could constitute an additional mechanism by which sleep fragmentation, characteristic of OSA, may promote cognitive decline and AD pathogenesis (see Fig. 2).

OSA causes REM sleep disruption

Although its complex functions are still incompletely understood, REM sleep has been implicated in sleep-related synaptic consolidation, neuroplasticity, and memory consolidation processes [83–86]. Muscular hypotonia is a characteristic of REM sleep, and a lower genioglossus muscle response in maintaining an adequate airway patency in this stage predisposes to apneic episodes. These episodes are in fact found to be more frequent, longer, and associated with greater hypoxemia in REM compared to N2 sleep stages [87–89]. The higher propensity for apneas during REM sleep in OSA could lead to a preferential disruption of this stage and its associated memory promoting processes. In older populations, REM sleep was found to be decreased in subjects with cognitive impairment compared to controls, which correlated with OSA severity [90]. A prospective 3-year follow-up study in older men corroborated

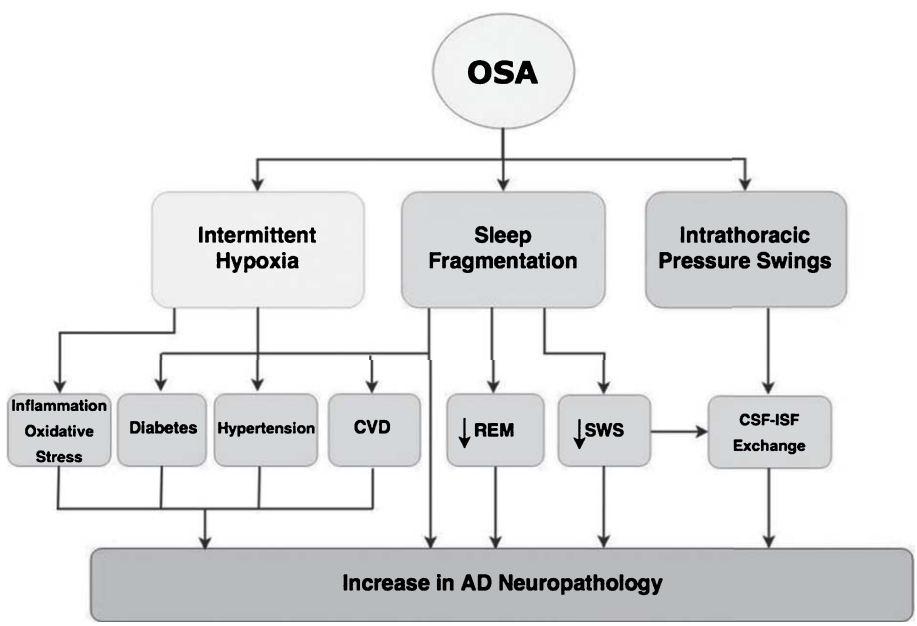

Fig. 2. Possible intermediate mechanisms in the relationship between OSA and AD. The effect of OSA in increasing the risk for AD can be mediated by several of its associated mechanisms. Chronic exposure to intermittent hypoxia may lead to increased inflammation and oxidative stress, diabetes, hypertension and CVD, all potentially contributing to AD pathology development. Sleep fragmentation, both by itself and by leading to decreased REM and SWS stages, can additionally promote AD pathogenesis. Finally, intrathoracic pressure swings associated with OSA may disrupt CSF-ISF exchange integrity and lead to AD neuropathology accumulation. OSA, obstructive sleep apnea; CVD, cardiovascular disease; REM, rapid eye movement; SWS, slow wave sleep; CSF-ISF, cerebrospinal fluid-interstitial fluid; AD, Alzheimer's disease.

that reduced REM stages were associated with greater cognitive decline over time [91]. Finally, a recent study in humans demonstrated that active and specific induction of OSA through CPAP withdrawal exclusively during REM sleep in patients with severe OSA resulted in spatial navigation learning deficits [92].

Several studies have additionally suggested a link between REM sleep disturbances and AD. At cross-section, AD patients had decreased REM sleep when compared to controls [93] and to depressed patients [94], although these findings were not replicated in other studies [95]. A recent prospective study on 321 subjects from the Framingham Heart Study cohort, examined the influence of PSG assessed sleep architecture features on the risk of AD. Lower total percentages and greater latencies to REM sleep at baseline associated strongly with AD incidence over a mean follow-up of 12 years, while all other sleep stages were not significantly associated with dementia risk [96]. The authors argued for a possible decrement in cholinergic activity known to accompany AD since early stages as a possible cause for this finding [97], but a primary role of REM reduction in AD pathogenesis could also be hypothesized. In this study, each percentage unit of REM sleep reduction was associated with a 9% increase in the risk of dementia, a value that was reduced to 6% when people with frequent arousals due to hypopneas were excluded. This suggested that OSA contributed to this observed association [96]. Additionally, a study using EEG detected frontal brain activity slowing, especially during REM sleep, in amnestic MCI compared to non-amnestic MCI and controls. This supports a possible impairment of REM sleep starting in the early clinical AD stages [98], however still without determining the causal direction of this relationship. Taken together, these studies point to a link between REM sleep, OSA, and AD. Whether OSA associated disruption of REM sleep contributes to cognitive decline and AD, or REM sleep disruptions are just (early) epiphenomena of AD is still unclear and more studies are required.

OSA causes SWS disruption

SWS is a stage of sleep that may be somewhat more resistant to OSA compared to lighter NREM stages [99]. This has been hypothesized to relate either to a greater upper airway stability being required for progression to deeper sleep stages [100, 101] or to an increased tolerance to hypoventilation during SWS leading to fewer arousals during this stage [102]. Nonetheless, it is clear that with increasing severity, OSA has the capacity to disrupt SWS. By selectively withdrawing CPAP exclusively in SWS in subjects with severe OSA, we found that there was both a reduction in %SWS and an increase in SWS fragmentation [103]. Guilleminault et al. reported a decrease in total SWS in older patients with severe OSA, both on the first NREM sleep cycle and on total night-time [104]. Another study with younger subjects and mild OSA, did not replicate this finding, however, a different time course of slow wave activity (SWA) was still found [105]. Additionally, severe OSA patients show up to a 40% homeostatic rebound in SWS duration following OSA treatment with CPAP, which suggest that changes in SWS quality are likely present in severe OSA [106].

OSA-induced reductions of SWS can be presumed to lead to cognitive impairment and increased AD risk for several reasons. First, SWS has been implicated in overnight memory [107], learning [108] and perceptual and visuomotor performance, all of which could be impaired in the presence of disturbances of this stage [109]. Second, neuronal activity is typically reduced during SWS, with an estimated decrement of up to 43% of glucose metabolism levels in ^{18}F-fluorodeoxyglucose (FDG) PET studies when compared to wakefulness [110]. Recent studies suggest that Aβ [111, 112] and tau release into the cerebral interstitial fluid (ISF) is increased during periods of higher synaptic activity, and that their clearance from this pool is higher during SWS [113]. SWS could be a beneficial stage due to both lower production of and increased removal of toxic metabolic byproducts. Corroborating this, our group recently found an association between reduced SWS and higher CSF levels of $A\beta_{42}$ [114]. Recently Ju et al., through SWS disruption with auditory tones, also found a strong association between SWS disruption and higher Aβ, and between lower sleep quality and increased tau CSF levels [115]. A possible decrease in SWS in OSA patients could therefore, by altering these production-clearance dynamics, predispose to AD.

EFFECTS OF VASCULAR COMORBIDITIES

OSA is associated with adverse cardiovascular outcomes

OSA is commonly accompanied by cardiovascular comorbidities. These include insulin resistance and diabetes, dyslipidemia, hypertension, and cardiac

diseases including dysrhythmias and congestive heart failure.

Epidemiological studies show that about half of type 2 diabetic patients are diagnosed with moderate or severe OSA and that approximately half of OSA patients have diabetes. Although both are highly prevalent disorders and a causal link is not yet proved, a bi-directional association between these conditions is suggested by some authors [116–118]. Insulin resistance was also found to correlate positively with OSA severity after controlling for potential confounders [10].

Dyslipidemia has been observed more frequently in OSA patients. In a prospective study, Chou et al. reported a prevalence of hypercholesterolemia and hypertriglyceridemia in OSA patients, of 61.1% and 55.3%, respectively [119]. A later randomized controlled trial study using CPAP demonstrated a reduction of postprandial lipidemia in OSA [11].

Hypertension is one of the best studied conditions accompanying OSA. OSA is common among hypertensive patients, with a global prevalence of 30% that increases up to 80% if only treatment-resistant cases are considered [2, 12]. On the other hand, as many as half of OSA patients have comorbid hypertension, and a systolic nondipping pattern of blood pressure during sleep is frequently observed in OSA [12, 120]. The causal weight of OSA on hypertension is nonetheless still debated and not as strong as originally thought. Conflicting conclusions were drawn from two large longitudinal studies, possibly due to age differences and the confounding effect of obesity, and a milder correlation between them is now suggested [121–123]. Reports from OSA clinical trials evaluating the effect of CPAP on hypertension are more convincing, with reductions of up to 2 mmHg in blood pressure, especially in cases of higher baseline hypertension and better compliance [124–126].

In OSA, both repetitive episodes of hypoxia and multiple arousals are thought to impair ventricular relaxation and myocardial contraction, contributing to the higher prevalence of ventricular hypertrophy and congestive heart failure in OSA [127]. Additionally, OSA results in recurrent decreases in intrathoracic pressure, by increasing left ventricular afterload and reducing pre-left ventricular load, which could also lead to reduction of ventricular ejection fraction [15, 128, 129]. Coronary heart disease has been inconsistently linked to OSA and more studies are required [2]. Cardiac arrhythmias, including atrial fibrillation, are frequent in OSA patients [130, 131], but whether they constitute a direct consequence of OSA or are mediated by heart failure is still debated [15]. CPAP treatment has been found to decrease the incidence of cardiovascular events [132].

Obesity is also frequently found in OSA patients and is suspected to be an important causal mechanism particularly in middle-aged adults, increasing also cardiovascular risk [133].

Finally, the incidence of stroke is higher in OSA patients [17, 18], and stroke, possibly due to its motor/respiratory sequelae, increases the risk for OSA. Prevalence of OSA in stroke patients rounds 50–70% and increases with recurrent strokes [134]. Some authors also suggest a bidirectional causal relationship between stroke and OSA [2].

Several mechanisms have been proposed to mediate the increased cardiovascular risk in OSA patients. These include sympathetic system activation [135, 136], oxidative stress [137, 138], local and systemic inflammation [139, 140], endothelial dysfunction, hypercoaguability [141, 142], and metabolic dysregulation (for a review, see [2]). Additionally, the effect of OSA on cardiovascular risk could be partially mediated by a decrease in SWS. Reduced SWS has been linked to metabolic, hormonal and autonomic disturbances [143, 144]. Interestingly, a prospective study in older men implicated SWS reduction but not OSA indices on hypertension risk [120], and in the same cohort, an inverse correlation between SWS and obesity was found [145].

Adverse cardiovascular outcomes increase risk of AD

Although all of these vascular and metabolic comorbidities could primarily contribute to vascular dementia [146], and not AD, a growing body of evidence is now attributing a pivotal role of cardiovascular disease in AD pathogenesis [147]. First, cardiac diseases such as atrial fibrillation, coronary heart disease, and heart failure, can directly lead to hypoperfusion and microemboli formation, which have been implicated in AD development [148–150]. Second, stroke can not only potentiate the clinical expression of AD [151], but several studies have shown that cerebral microinfarcts and intracranial atherosclerosis can increase the risk of AD [152, 153]. It has been proposed that cerebrovascular disease could directly promote Aβ production and reduce its clearance [154, 155]; however, available data on this hypothesis is still inconsistent [155, 156]. Besides its accepted implication in neuropathic

cerebrovascular mechanisms, hypertension in midlife has been directly associated with a higher development of neuritic plaques, NFTs, and brain atrophy, suggesting another link to AD pathogenesis [157, 158]. Both type 2 diabetes and pre-diabetes have been shown to increase the risk of dementia and AD, possibly due to microvascular damage and neurotoxicity of higher levels of glucose and insulin leading to oxidative stress [159, 160]. A cross-sectional study in 156 patients with incident AD, documented an association between pre-diagnosis dyslipidemia (higher total and LDL cholesterol) and diabetes, and faster cognitive decline. This association seems to be conditioned by ApoE4 status, as a previous history of stroke or heart disease was associated with cognitive deterioration only in ApoE4 carriers [161]. In conclusion, although it is more commonly accepted that vascular and metabolic OSA associated comorbidities may lead to stroke and vascular dementia, an alternative role of cerebrovascular pathology in AD pathogenesis is now recognized, with both pathologies synergistically promoting cognitive decline [162]. Finally, midlife obesity, possibly due to its association with many chronic vascular diseases, has been documented to increase the risk of dementia and AD [163]. Together, these data suggest that OSA associated vascular and metabolic comorbidities could, through chronic impairment of cerebrovascular integrity and/or neurometabolic systems, lead to an increased risk of AD.

AD PATHOLOGY IS ASSOCIATED WITH INTERMITTENT HYPOXIA AND OXIDATIVE STRESS

Oxidative stress is caused by an imbalance between the production and clearance of reactive oxygen species (ROS) [2]. These oxygen-rich molecules are highly reactive with proteins, lipids, and nucleic acids, and have been implicated in neuronal dysfunction and death in neurodegenerative diseases [2, 164]. Mounting evidence suggests that repetitive cycles of intermittent hypoxia followed by reoxygenation, characteristic of OSA, promote ROS production [137, 138] and reduce blood antioxidant capacity [165]. In humans, OSA is associated with higher systemic biomarkers of oxidative stress and inflammation, that parallel disease severity [166]. This intermittent hypoxia-induced oxidative stress effect has been hypothesized to underlie, at least partially, cognitive changes in OSA [24]. In fact, several studies in rodents, have shown that intermittent hypoxia during rest is associated with increased oxidative stress and inflammation biomarkers, increased neuronal loss and reduced spatial learning [167–169]. This deleterious effect was shown to be reduced by the use of pharmacological inhibitors of oxidative stress pathways [164]. Baril et al. recently demonstrated a thickening of gray matter paralleling OSA severity [170], which they hypothesized to stem from edema [171] and reactive gliosis [172] associated with hypoxemia.

Some studies further suggest a contribution of intermittent hypoxia to AD pathophysiology. Ng et al. showed that short-term chronic intermittent hypoxia increased Aβ peptide generation in rat hippocampi and that this effect was prevented by melatonin administration [173]. A study using neuronal culture from triple transgenic AD mice documented a significant increase in $A\beta_{42}$ in brain cortex associated with intermittent hypoxia, both supporting a role of OSA in AD progression [27]. Furthermore, there is evidence of tau-phosphorylation activation with chronic hypoxia in double transgenic (APP/PS1) mice [28], increases of CSF and serum T-tau after cardiac arrest [174], and increases in P-tau in hypertensive patients with blood pressure reductions in possible relation with hypoperfusion [175]. A large clinical longitudinal study confirmed an association between measures of OSA and incidence of MCI and dementia in older women, and this effect was attributed to hypoxemia effects rather than sleep fragmentation or duration [30]. In summary, growing evidence shows that intermittent hypoxia in OSA can be an important factor contributing to an increased risk of cognitive decline and AD progression in these patients.

OSA IS ASSOCIATED WITH DECREASED CSF-ISF CLEARANCE

The respiratory effort against collapsed airways during OSA apneic episodes (Mueller maneuver) is associated with elevated intrathoracic and intracranial pressures, and hemodynamic disturbances [176, 177]. These have been hypothesized to acutely and repetitively impede the circulation of brain metabolites from ISF into CSF [178], through the glymphatic system, leading to increased $A\beta_{42}$ accumulation in the ISF. This mechanism was proposed by a recent study where all assessed CSF neuronally derived proteins, but not total protein (mainly derived from blood

albumin), were decreased in severe OSA subjects compared to controls [178], suggesting that clearance glymphatic processes were impaired in OSA. As an alternative, the authors proposed that an increased venous pressure seen in OSA due to intermittent hypoxia and right heart strain could limit the clearance of subarachnoid CSF into the dural lymphatic system, leading to the reduced concentrations of metabolites observed in the CSF [178, 179]. Another possible pathway for CSF-ISF exchange impairment in OSA could be cerebral edema secondary to intermittent hypoxia as described previously. In this study, severity of OSA correlated with increased volume and thickness of the left lateral prefrontal cortex, as well as increased thickness of the right frontal pole, the right lateral parietal lobules, and the left posterior cingulate cortex [170]. In a previous interventional study, these findings were found to reverse after six months of treatment with CPAP, suggesting the existence of brain edema in OSA [180]. In conclusion, decreased clearance of amyloid is believed to be one of the mechanisms underlying AD pathogenesis and could be affected by mechanical and brain localized OSA changes, comprising an additional pathway through which OSA could contribute to increased AD risk (Fig. 2).

AD CAN CONTRIBUTE TO OSA

The characteristic progressive brain accumulation of amyloid plaques and NFTs in AD may determine changes in sleep patterns, sometimes even before overt dementia is recognized. A reduction in SWS is frequently observed in AD patients and since this stage is associated with fewer apneic events [102], this could lead to increased OSA severity in AD patients. Relatedly, lighter sleep stages as N1 and N2 NREM prevail in AD subjects. As these stages are associated with a higher propensity for apneas, this may also generate a trend toward worsening of OSA severity in AD [99]. Additionally, potential age-dependent anatomical [181] and functional neuromuscular [182] upper airway changes that affect nocturnal respiratory patency, may be aggravated in AD patients. Either through accumulating pathology or neuronal loss, both gray matter and white matters structures responsible for motor response can be affected in AD patients [183], potentially increasing their susceptibility for OSA. Taken together, all these mechanisms could render AD as a risk factor for OSA. Ultimately both diseases could have a bidirectional and cyclic potentiating effect on each other's pathogenesis.

CONCLUSION

Although it is known that OSA is a highly prevalent disease with growing impact in our society, data from epidemiological studies is still lacking the consistency and strength to fully understand its relationship to frequently associated comorbidities and mortality, especially in milder forms of the disease. Its classification based only on AHI cutoffs seems to be now too simplistic, as OSA appears to be a more complex and heterogeneous disorder, continuously interacting with aging, other risk factors, and its own comorbidities. The leading contributing causes for OSA in the young, as craniofacial predisposing morphology, obesity, family history, and male sex, may differ from the ones in the elderly, where the impact of possible anatomical, functional, and sleep architecture changes determined by the aging process seems to prevail. Improvements in epidemiologic study design that may promote a better understanding of this pressing issue and necessary advancements in the field are currently being discussed and proposed [184].

Multiple lines of evidence suggest that OSA potentiates neuropathological and clinical progression of AD. Probably by a combination of mechanisms including disruption of sleep architecture, intermittent hypoxia, and hemodynamic changes, and the deleterious effects of its vascular comorbidities, OSA may determine a cumulative predisposing context for AD development. While AD does not have an effective treatment, several pathologic mechanisms in OSA can be reverted by OSA treatment, including correct and sufficient CPAP use, and exploring this relationship may converge in possible manipulations of this risk factor to help prevent cognitive decline and dementia.

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From Cerebrospinal Fluid to Blood: The Third Wave of Fluid Biomarkers for Alzheimer's Disease

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Abstract. The past five years have seen an enormous development in the field of fluid biomarkers for Alzheimer's disease (AD) and related disorders. The proteins that constitute the foundation for the cerebrospinal fluid (CSF) tests for the classical AD pathologies are now being explored as potential blood-based biomarkers, thanks to the recent implementation of ultrasensitive measurement technologies in academic and clinical laboratories worldwide. The current blood-derived data are still less clear than those obtained using CSF as the sample type, but independent research suggests that there are biomarker signals in blood that relate to plaque and tangle pathologies in AD, which are relevant to explore further. Additionally, neurofilament light has emerged as the first robust blood-based biomarker for neurodegeneration in a broad range of central nervous system disorders, as well as for acute brain injuries. Here, we briefly recapitulate the first and second waves of fluid biomarker analysis in AD, i.e., the development and validation of established and novel CSF biomarkers for the disorder, followed by a focused discussion on blood-based biomarkers for AD, which we describe as the third wave of fluid biomarker analysis that hopefully will gain further momentum during the coming five years.

Keywords: Alzheimer's disease, amyloid, biomarkers, cerebrospinal fluid, plasma, serum, tau

INTRODUCTION

The best established fluid biomarkers for Alzheimer's disease (AD) are cerebrospinal fluid (CSF) concentrations of total tau (T-tau), phospho-tau (P-tau), and the 42 amino acid form of amyloid-β ($A\beta_{42}$) [1]. The discovery and validation of these biomarkers and the development of robust tests for them may be described as the first wave of fluid biomarker analysis in AD research. During the past five years, it has been confirmed that CSF $A\beta_{42}$ indeed is a reliable marker of amyloid (plaque) pathology in the brain (as determined at autopsy or through amyloid positron emission tomography [PET] studies), especially when measured in a ratio with CSF $A\beta_{40}$ [2]. For CSF T-tau and P-tau, the interpretation is less clear; tau markers are robustly increased in AD CSF [1], but the exact mechanism remains unclear, especially for P-tau [3].

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Some data suggest that neurons exposed to Alzheimer-associated factors such as Aβ may increase their secretion of both tau proteins [4]. Neurons who respond in this way may eventually accumulate tau (or tangle) pathology and degenerate. In spite of these uncertainties, the diagnostic performance and clinical utility of CSF T-tau, P-tau, and $A\beta_{42}$ are undisputed: new diagnostic algorithms including CSF biomarkers have been formulated [5], automated routine clinical chemistry assays for the markers are now becoming available [6], and standardization efforts to harmonize assays are well underway; reference methods for $A\beta_{42}$ have been formally certified by the Joint Committee for Traceability in Laboratory Medicine (JCTLM database accession numbers C11RMP9 and C12RMP1) [7, 8] and validated against amyloid PET [9], and a reference material for CSF $A\beta_{42}$ is soon to be certified and released [10]. Similar work is ongoing for CSF tau biomarkers.

During the past five years, a number of additional CSF biomarkers for AD-related pathological processes have become available (the second wave). These include neurofilament light (NF-L) as a marker of neurodegeneration [11], neurogranin (Ng) as a marker of synapse dysfunction and/or loss [12], and sTREM2 and YKL-40 as markers of microglial and astrocytic activation [13, 14]. These biomarkers have been extensively reviewed elsewhere [1] and updated meta-analyses regarding their association with AD can be found in the AlzBiomarker database (http://www.alzforum.org/alzbiomarker).

Here, we will focus on the third wave of fluid biomarker analysis in AD: the development of blood-based biomarkers for AD-related pathologies, which we believe will gain further momentum during the coming five years.

METHODOLOGICAL CONSIDERATIONS

Blood as a biomarker matrix

Whereas CSF is a well-established sample type for the analysis of biomarkers for neurodegenerative diseases (it communicates freely with the brain interstitial fluid that bathes the neurons and has relatively low turnover and protease activity), blood has emerged more recently after decades of relatively disappointing results. Blood communicates with the brain across the blood-brain barrier, via lymph vessels [15] and through the glymphatic system [16]. This interchange, however, is less direct than for CSF and there are several challenges, both biological and technical, with the measurement of central nervous system (CNS)-related biomarkers in blood. First, a biomarker that has its origin in the CNS has to cross the blood-brain barrier in order to be detected in the periphery and, if the concentration is low in CSF, it will be even lower in the blood due to the blood:CSF volume ratio causing a substantial dilution of the analyte. Second, if the biomarker is not specific for the CNS but also expressed in peripheral tissues, the contribution from CNS will potentially drown in the high biological background caused by non-CNS sources (a good tool to assess the risk for this is the publicly available web-based Human Protein Atlas, http://www.proteinatlas.org/, which presents mRNA and protein expression in 44 different human tissues of close to 20,000 proteins) [17]. Third, the huge amount of other proteins in blood (e.g., albumin, immunoglobulins, α1-antitrypsin, transferrin, haptoglobin, and fibrinogen) introduces analytical challenges due to possible interference [18]. Fourth, heterophilic antibodies may be present in blood, which may interfere in immunoassays [19], while the levels of these are much lower in CSF samples. Fifth, the analyte of interest may undergo proteolytic degradation in plasma and clearance in the liver or by the kidneys that may introduce variation [20]. Finally, there may be additional pre-analytical factors that may be more relevant for blood- than CSF-based biomarkers, including diurnal variation and influences of, for example, food intake and medication.

Ultrasensitive measurement techniques

Many, but not all, of the challenges reviewed above may be overcome with more sensitive assays with adequate blocking of heterophilic antibodies and improved pre-analytical standardization. Most biomarker assays of relevance to AD are immunochemical, i.e., utilize antibodies to quantify a substance in a sample. The most common assay format is the sandwich enzyme-linked immunosorbent assay (ELISA) in which the target analyte is captured between two antibodies in a complex and one of the antibodies carries a signal generator, i.e., an enzyme that converts a substrate into a detectable form (colored, fluorescent, or luminescent), which, in combination with a calibrator curve (derived from artificial samples with known analyte concentrations), allows for quantification of the analyte of interest. ELISA is a theme with many variations, such as the choice of signal generator where the enzyme

can be replaced by, e.g., a fluorophore or a DNA-based detection system.

The technical issues are mainly a question of antibody sensitivity and specificity. In theory, if the time for the enzyme reaction is simply extended, this should increase the sensitivity of the assay. However, the substrates used are inherently unstable and therefore produce signal even in the absence of enzyme. This leads to a technical background signal that can mask the signal generated by the sandwich complex, making quantification uncertain at low concentrations. In the end, the ability of the sandwich complex to correctly represent the concentration of the biomarker in a sample strongly depends on the quality of the antibodies used. If the antibodies cross-react with other substances, a signal can be measured even in the absence of the target analyte. Since the blood is much denser in protein content than is CSF, the risk for this is higher in the former, where even minor (e.g., 0.1%) cross-reactivity against proteins present at one million times higher concentrations will have a large impact on the measured concentration.

Most of the ultrasensitive technologies rely on antibody-based detection of the target molecule, but in Single molecule array (Simoa), the detection reaction is compartmentalized into a small volume (50 femtolitres), so that the reporter molecule accumulates at a very high concentration [21]; in Single molecule counting (SMC), the labelled detection antibodies, specifically captured by the target molecule/capture antibody complex, are released and counted one by one in a small detection cell, which allows for a single molecule read-out [22]; and in proximity extension assay (PEA), partly overlapping complementary DNA strands are attached to the different antibodies allowing the strands to form a polymerase chain reaction-amplifiable template if immobilized close to each other on the same molecule [23]. These variations in signal generation/detection may result in assays that can be 10- to a 1000-fold as sensitive as the corresponding regular ELISA using the same antibody pair.

Mass spectrometry (MS)-based assays are increasingly important in clinical laboratory medicine, mostly to measure small molecules, such as drugs, amino acids, hormones, and vitamins in an antibody-independent manner [24]. Mass spectrometers are also used in explorative proteomics studies to identify new biomarker candidates. However, explorative proteomics has so far failed to generate validated AD biomarkers and, in general, MS-based standardized quantification of peptides and proteins for routine diagnostic use remains rare [25]. However, this is changing and for example Aβ can be reliably quantified in plasma using immunoprecipitation and matrix-assisted-laser-desorption/ionization time-of-flight/time-of-flight mass spectrometry [26, 27].

BLOOD-BASED BIOMARKERS FOR AD-ASSOCIATED PATHOPHYSIOLOGICAL PROCESSES

Blood-based biomarkers for amyloid pathology

It has been difficult to establish robust blood biomarkers for Aβ pathology in AD. Aβ proteins can be measured in plasma but historically the correlation with AD and/or cerebral β-amyloidosis has been absent or weak (statistically significant but clinically meaningless) [1]. Plasma Aβ concentrations have been interpreted as potentially influenced by production in platelets and other extra-cerebral tissues and the measurements have been confounded by matrix effects from plasma proteins [28]. However, this view is now starting to change. Recent mass spectrometric studies suggest that a ratio of a certain amyloid-β protein precursor (AβPP) fragment (AβPP669-711; an Aβ peptide that extends over the BACE1 cleavage site of AβPP with 3 amino acids), to $A\beta_{42}$ or $A\beta_{42}/A\beta_{40}$ identifies individuals cerebral β-amyloidosis with high sensitivity and specificity [26, 29]. The latter result is in line with earlier data obtained using ultrasensitive Simoa technology by which the sample can be diluted to remove confounding matrix effects in the Aβ measurement [30]. Pilot data suggest associations of the concentrations of a number of plasma proteins (e.g., pancreatic polypeptide Y, IgM, chemokine ligand 13, interleukin 17, vascular cell adhesion protein 1, α2-macroglobulin, apolipoprotein A1, and complement proteins) with amyloid burden in the brain [31–33]. However, these data should be interpreted with some caution, as they are derived from multi-marker panels and as a mechanistic understanding of the associations is currently lacking.

Blood-based biomarkers for tangle pathology

There are so far no validated blood biomarkers for neurofibrillary tangle pathology, although there is an emerging literature on P-tau concentrations in neuronally derived blood exosomes with varying results

in regards to the association with AD [34, 35]. A recent study employed Simoa technology to measure P-tau phosphorylated at amino acid 181 in plasma (without exosomal enrichment) from AD patients ($n = 28$), individuals with Down's syndrome (DS, $n = 20$), and matched controls ($n = 15$) [36]. The mean plasma P-tau concentration was about 3-4-fold higher in AD patients and DS individuals than in controls, but the numbers in each group were too small to determine the diagnostic accuracy of the test with certainty (pilot receiver operating characteristics curves suggested optimal sensitivity and specificity of 60% and 86%, respectively, for the AD-control comparison). Importantly, however, plasma P-tau correlated with CSF P-tau concentration in a sub-cohort composed of 8 AD patients and 3 patients with other neurological diseases. In another recent paper, plasma P-tau (phosphorylated at amino acid 231) was measured in patients with traumatic brain injury (TBI) using a fiber optics technique in which antibody-based detection was combined with rolling circle amplification to increase the analytical sensitivity so that P-tau could be quantified in most samples [37]. Increased concentrations of plasma P-tau in TBI patients were reported but no data on AD was presented. Taken together, plasma P-tau is a hot topic in AD biomarker research and it will be interesting to follow how it develops during the coming five years.

Blood-based biomarkers for neurodegeneration

CSF assays for T-tau and NF-L were recently developed into ultrasensitive blood tests using Simoa technology [38]. Serum or plasma NF-L concentration (either sample matrix works well) correlates with CSF (correlation coefficients of 0.75 to 0.97) and most CSF findings (increased NF-L concentrations in AD, frontotemporal dementia, vascular dementia, and atypical parkinsonian disorders) have been replicated in blood [11]. Recent data show that serum NF-L effectively identifies onset of neurodegeneration in familial AD [39] and Huntington's disease [40]. Plasma NF-L concentration is increased in patients with Charcot-Marie-Tooth disease and correlates with disease severity, suggesting that peripheral nerves may also release NF-L [41]. This could potentially smudge the association of plasma NF-L with central axonal degeneration, but the robust association of plasma/serum NF-L with CSF NF-L suggests that most of the NF-L signal in blood is CNS-derived [42–44], at least in the absence of significant peripheral nerve disease.

For tau, the situation is promising but less clear. Firstly, for unknown reasons, tau concentrations are higher in plasma than in serum (unpublished observation). Secondly, the correlation with the corresponding CSF concentration is absent [45] or weak [46]. Plasma T-tau concentration in AD is increased but the effect size is smaller than in CSF and there is no detectable increase in the mild cognitive impairment (MCI) stage of the disease [45, 46]. In a recent paper, Mielke and colleagues examined the relationship of plasma T-tau concentration, determined by Simoa, with cognitive decline in 458 participants from the Mayo Clinic Study on Aging [47]. Included subjects were cognitively normal at baseline and followed for up to 4 years. Plasma T-tau correlated with cognitive decline in the sense that higher plasma levels in both the cognitively normal and MCI groups predicted steeper decline in global cognition, memory, attention and visuospatial ability over three years. During follow-up, 67 of 335 cognitively normal people developed MCI. Those in the highest and middle tertiles of plasma t-tau were likelier to progress than those in the lowest. Over that same period, 28 of 123 people with MCI progressed to dementia, however, plasma T-tau did not predict who would. Altogether, the published studies on plasma T-tau as an AD biomarker so far point toward the feasibility of finding a predictive tau signal in blood. However, the lack of correlation of plasma with CSF T-tau suggests that researchers should look for additional tau biomarkers in plasma, e.g., degradation end-products that may be more stable and potentially reflect CNS tau better.

In regards to synaptic degeneration in AD, CSF neurogranin has emerged as the most promising fluid marker [48–53]. However, when examined in plasma, neurogranin is unchanged in AD and there is no correlation with CSF, most likely due to expression in peripheral tissues [54].

Blood-based biomarkers for microglial activation

Recent reports suggest that the CSF concentration of the secreted ectodomain of triggering receptor expressed on myeloid cells 2 (Trem2), a molecule that is selectively expressed on microglia in the CNS [55, 56] and genetically linked to AD [57, 58], is increased in AD in a disease-specific manner and correlates with CSF T-tau and P-tau [59–61]. These results are backed by an abundant literature showing increased CSF concentrations of several other microglia- and/or macrophage-derived proteins, including chitotriosidase [62, 63], CD14 [64],

and YKL-40 [65, 66]. Another microglial marker, the C-C chemokine receptor 2, is expressed on monocytes and one of its ligands, C-C chemokine ligand 2 (CCL2), that can be produced by microglia, is present at increased concentration in AD CSF [67–69]. Most studies suggest that these increases are modest with large overlaps between cases and controls, if compared to the more prominent changes seen in traditional neuroinflammatory conditions, such as multiple sclerosis [70] or HIV-associated neurocognitive dysfunction [71]. When measured in blood, the concentrations of most of the microglia-related proteins mentioned above are higher than in CSF and probably reflect release from monocytes and macrophages in peripheral blood rather than CNS-related changes. However, a few studies suggest a slightly increased concentration of YKL-40 in plasma from AD patients [1].

Blood-based biomarkers for AD-associated protein accumulations other than tau and Aβ

α-Synuclein is the major component of Lewy bodies that are characteristic inclusions of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [72] but often also seen in AD [73]. In PD and other synucleinopathies, CSF α-synuclein concentrations are typically lower than in controls [74, 75], while in AD and Creutzfeldt-Jakob disease, the concentrations are increased and correlate with T-tau, suggesting that α-synuclein may also be an non-specific marker of neurodegeneration [75–79]. This has been reported not only in AD and Creutzfeldt-Jakob disease, but also in DLB, where there may be a competition between aggregation of α-synuclein into Lewy bodies and release of the protein from degenerating synapses, making the data complex to interpret [80]. Currently available assays for α-synuclein measure total amounts of the protein and not Lewy body-specific isoforms; sensitive and specific assays for the latter would resolve this issue. However, there are some preliminary reports on increased CSF concentrations of α-synuclein oligomers in CSF from PD patients [81, 82] and recently sensitive assays that detect and amplify the biochemical signal of what appears to be α-synuclein seeds in CSF have been published [83, 84]. α-Synuclein is highly expressed in red blood cells, a reason why blood contamination during CSF collection may limit the diagnostic value [85, 86]. For the very same reason, blood tests for α-synuclein pathology in the brain may prove hard to develop. Nevertheless, as peripheral Lewy body pathology, e.g., in the salivary gland and gut, has been reported in PD [87], blood or salivary tests for α-synuclein seeds may be something to explore in the future.

Another pathology that commonly co-occurs with classical AD pathology is inclusions of hyperphosphorylated transactive response DNA-binding protein 43 (TDP-43) [88], traditionally linked to frontotemporal dementia. TDP-43 can be measured in CSF but, unfortunately, most of the protein appears to be blood-derived and its CSF concentration does not reflect TDP-43 pathology and is unaltered in frontotemporal dementia [89]. Similarly, no reliable blood test for TDP-43 pathology in the CNS exists to date, but intense research efforts are ongoing.

Miscellaneous

There is vibrant research activity on other potential AD biomarkers, such as exosomes and micro-RNA, lipid and metabolite profiles, using both CSF and blood as sample types in explorative studies. These are still in their infancy but may well represent an emerging fourth wave of AD biomarkers during the coming five years.

CONCLUDING REMARKS

The past five years have seen an enormous development in analytical tools for ultrasensitive biomarker quantification in the context of neurodegenerative diseases. The development in the field has been much faster than we ever could have imagined. Assays that are 100- to 1000-fold as sensitive as standard ELISA or mass spectrometry-based techniques have opened up a new biomarker window in the CSF and made it possible to quantify the traditional CSF biomarkers in blood. NF-L is the only CSF biomarker for which the transition from CSF to blood has been relatively uncomplicated, but for tau and Aβ biomarkers, there is a signal also in blood, albeit with a smaller effect size than what can be obtained using the corresponding CSF measure. We believe that new ultrasensitive techniques will allow for the development of assays for the quantification of fragments or protein subforms that are more stable in blood and/or more sensitive and specific to CNS pathologies. This will hopefully lead to more robust blood-based assays that eventually could be used as diagnostic and/or screening tools also in primary care. During the coming years, it will be important to continue to build biobanks from deeply phenotyped cohorts with

access to both CSF and blood samples, as well as data on advanced neuroimaging, genetics, and clinical follow-up. This should facilitate the development of even better tests, which will be particularly useful the day we have the first disease-modifying treatment. At present, we do not think blood-based analysis will substitute CSF analysis, but perhaps sequential testing, starting with blood analysis followed by referral of selected patients for CSF analysis and additional examinations at expert centers will be the future.

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Cerebrospinal Fluid Biomarkers in Alzheimer's Disease: An Invaluable Tool for Clinical Diagnosis and Trial Enrichment

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Abstract. Alzheimer's disease (AD) is the most common neurodegenerative disorder, affecting around 35 million people worldwide. Cerebrospinal fluid (CSF) biomarkers entered the diagnostic criteria as support for early diagnosis. The classical biochemical signature of AD includes total tau (T-tau), phosphorylated tau (P-tau), and the 42 amino acid peptide ($A\beta_{42}$) of amyloid-β. Recent observations suggest that the use of CSF $A\beta_{42}$:$A\beta_{40}$ ratio rather than CSF $A\beta_{42}$ alone could contribute to reduce inter-laboratory variation in Aβ values and increasing diagnostic performance of the CSF AD biomarkers in routine practice. However, research efforts aimed at enriching the CSF biomarker panel are ongoing. The CSF AD signature is also crucial for the design of clinical trials for AD, since it best guarantees AD pathology as the cause of cognitive impairment. Accordingly, CSF biomarkers have been now reported in the inclusion criteria of Phase I, Phase II, and Phase III clinical trials as enrichment strategy. So far, one of the most important reasons for the failure of AD clinical trials was the inclusion of participants with unlikely AD pathology. In order to implement the use of CSF biomarkers in AD routine diagnostic work-up and as accepted strategy for enriching trial populations, inter-laboratory variability should be minimized. Increasing efforts should also be devoted to promote data sharing practices, encouraging individual participant data meta-analyses.

Keywords: Alzheimer's disease, amyloid, cerebrospinal fluid biomarkers, early diagnosis, tau

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder, affecting around 35 million people worldwide. Currently, available treatments for this disorder aim to reduce symptoms, and do not have detectable effects on disease progression. Evidence indicates that there is a long preclinical and prodromal phase before the full-blown syndrome appears.

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Therefore, ideal disease-modifying pharmacological treatments should be administered as early as possible, that is, before the neurodegenerative process becomes too severe and widespread [1]. This is one of the most compelling reasons for searching an early AD signature based on cerebrospinal fluid (CSF) biomarkers. However, even if disease-modifying therapies are lacking, the advantages of an accurate diagnosis justify the use of advanced diagnostic technology [2]. In fact, an accurate early diagnosis of AD before the onset of dementia is vital to ensure that patients receive timely and appropriate personalized care, including counseling and planning, avoiding the use of inappropriate medications

and ancillary investigations. Furthermore, it allows the eventual implementation of appropriate steps to prevent unsafe behaviors also allowing the patients to decide and manage legal issues, being still competent. Besides its utility in clinical practice, the availability of a CSF based toolbox for the early diagnosis of AD is pivotal to the development of better strategies for patient recruitment in research studies and clinical trials.

CSF BIOMARKERS IN ROUTINE DIAGNOSTIC WORK-UP

The core AD CSF biomarkers include total tau (T-tau), phosphorylated tau (P-tau), and the 42 amino acid peptide ($A\beta_{42}$) of amyloid-β. These proteins reflect key pathogenic aspects of the disease, i.e., neuronal and axonal degeneration, phosphorylation of tau with tangle formation, and aggregation and deposition of the $A\beta_{42}$ peptide into plaques [3].

After being validated in several studies and meta-analyses [4], CSF biomarkers entered the AD diagnostic criteria. In the International Working Group IWG-2 criteria [5], CSF biomarkers have a pivotal role for the diagnosis of prodromal AD, together with amyloid PET, because of their high diagnostic performance.

The National Institute on Aging–Alzheimer's Association (NIA-AA) criteria for mild cognitive impairment (MCI) due to AD [6] and dementia due to AD [7] allow for the assessment of the likelihood of being correctly diagnosed with both amyloid and (neuronal) injury biomarker, with positive cases having the highest likelihood. Although the two criteria sets are based upon different approaches and terminology, most patients who meet the IWG-2 criteria will also meet the NIA-AA criteria and vice versa [8].

The NIA-AA criteria for MCI due to AD consider both amnestic and non-amnestic MCI as possible prodromal stages of AD-type dementia. Several studies showed that CSF biomarkers predicted accurately AD dementia in subject with amnestic MCI [9–11].

In recent years, several studies have pointed out the utility of including $A\beta_{40}$, the most abundant variant of Aβ isoforms, in the CSF signature of AD. Even if CSF $A\beta_{40}$ is relatively unchanged in AD, the CSF $A\beta_{42}$:$A\beta_{40}$ ratio has been suggested to have stronger diagnostic accuracy for AD when compared with CSF $A\beta_{42}$ alone [12, 13]. Accordingly, many reports show that the CSF $A\beta_{42}$:$A\beta_{40}$ ratio is more closely related to what is observed with PET amyloid imaging [14]. Furthermore, a recent finding also suggests that the use of the CSF $A\beta_{42}$:$A\beta_{40}$ ratio rather than CSF $A\beta_{42}$ alone could contribute to reduce inter-laboratory variation in Aβ values, thus favoring the general use of CSF AD biomarkers in routine practice [15].

A recent paper outlined a strategic roadmap for bridging the gaps until an early AD diagnosis based on biomarkers (i.e., CSF or imaging) will be completely integrated in clinical practice [16]. The roadmap is built upon the development and use of biomarkers for screening and delivery of personalized care in oncology, a discipline offering a unique perspective, due to the most advanced stages of implementation of biomarkers for prevention, diagnosis, and treatment. As in the framework developed for oncological patients in 2001 by Pepe and colleagues [17], the roadmap includes five phases characterized by one or two primary aims, as well as several secondary objectives. According to this approach, we can see that CSF biomarkers for AD are at an advanced stage of development [18].

A major drawback for CSF biomarkers is that the measurements obtained with currently available manual immunoassays are sufficiently stable and comparable only when used in experienced laboratories with well-established quality control procedures. Important recent advancements are represented by automated assays [19, 20], which in the near future will significantly increase precision by minimizing operator errors, potentially allowing a greater diffusion of CSF analysis also in non-research centers.

Nevertheless, standardized protocols for controlling pre-analytical and analytical factors remain a priority. We need to reassess the cutoff values for all immunoassays by using a suitable reference (preferably neuropathology). Despite large evidence from studies of clinical assay development and observations on retrospective studies using longitudinal data available in repositories, there is still modest evidence in prospective diagnostic accuracy studies and none in disease burden reduction studies.

NEW CSF BIOMARKERS TO EMPOWER THE DIAGNOSTIC PERFORMANCE OF THE AD BIOCHEMICAL SIGNATURE

The CSF Alzheimer signature is represented by increased total tau (T-tau) and phosphorylated tau (P-tau), together with reduced $A\beta_{42}$ and $A\beta_{42}$:$A\beta_{40}$

ratio. However, several observations in the last years have shown that there is room to improve this well established and in-use toolbox. A recent meta-analysis by Olsson and colleagues [4] found that, within the large number of CSF biomarkers studied so far, neurofilament light protein is also strongly associated with AD. Other molecules not directly reflecting AD core pathology, namely, neuron specific enolase (NSE) [21], a neuron-enriched enzyme of the glycolytic pathway, visinin-like protein 1 (VLP-1), a calcium-sensor protein found in the neuronal cytoplasm [22], heart fatty acid binding protein (HFABP), an intracellular fatty acid transport protein also expressed in neurons [23], and YKL-40 a marker of activated microglia and astrocytes [24, 25], may add accuracy for diagnosing AD. These molecules could be promising candidates as prognostic markers, as well.

THE ROLE OF CSF BIOMARKERS FOR TRIAL ENRICHMENT

Bapineuzumab and solanezumab Phase III trials in mild to moderate AD ended up with negative results. One possible reason for that is the inclusion of participants with unlikely AD pathology [26].

PET sub-studies of bapineuzumab and solanezumab trials classified the patients that were amyloid-negative (Aβ-) based on amyloid PET imaging and demonstrated that more than 20% of patients diagnosed with AD based on clinical criteria were Aβ-, with higher proportions of Aβ- among APOE ε4 non-carrier and mild dementia patients [27]. As expected, Aβ- subjects did not demonstrate the same rate of cognitive decline typically observed in AD. These findings, along with other observations, show the basic need of β-amyloidosis markers, either PET amyloid imaging or CSF Aβ levels, for the purpose of trial enrichment.

As reported above, PET amyloid imaging or CSF Aβ levels are used in the new NIA-AA criteria for evidentiating brain β-amyloidosis. Accordingly, many ongoing or planned trials are using these amyloid biomarkers as enrichment to catch prodromal AD cases.

Coric and colleagues [28] reported the results of a randomized, placebo-controlled phase II clinical trial that prospectively enriched a study population with prodromal AD defined by CSF biomarker criteria and MCI symptoms. The study failed to demonstrate clinically meaningful pharmacodynamic effects of avagacestat but met its clinical trial enrichment aims.

CSF biomarkers have been now reported in the inclusion criteria of Phase I, Phase II, and Phase III clinical trials, with enrichment strategy pursued in several manners (Table 1).

In trials on AD populations, several definitions are used to list CSF AD markers in the inclusion criteria: to meet NIA-AA criteria; to have a CSF profile consistent with AD pathology; CSF $A\beta_{42}$ under a certain cut-off depending of the target population; to lie below/above of $A\beta_{42}$/tau cut-offs. In trials including MCI patients, participants are required to meet NIA-AA criteria for MCI due to AD.

The use of CSF $A\beta_{42}$ and tau proteins as inclusion criterion for clinical trials in patients with AD has been endorsed by the European Medicines Agency (EMA). The EMA released two qualification opinions, in April 2011 and February 2012, stating that a pathological signature based on low CSF $A\beta_{42}$ and high t-tau levels in patients with MCI is useful for identifying those who are at risk of developing AD dementia. In addition, given the high sensitivity and moderate specificity, EMA concluded that the CSF biomarker signature based on a low $A\beta_{42}$ and a high T-tau is useful for the enrichment of clinical trial populations [29]. The FDA has also released draft guidance on clinical trials in patients in the predementia stage of AD. According to this guidance, FDA supports the concept of enriching trial populations with patients most likely to progress to dementia, using both clinical and biomarker-based criteria. However, the need for an assessment of sensitivity and specificity in identifying patients who do have actual AD in clinical trials, as well as for the validation methodologies (e.g., selection of appropriate cut-points, quantification of assay variability), does not allow FDA to formally endorse CSF biomarkers as definite diagnostic tool, at this time.

To date, the FDA has issued a letter of support to Coalition Against Major Diseases encouraging the further use and study of CSF analytes as exploratory prognostic biomarkers for enrichment in clinical trials targeting the pre-dementia stage of the disease [30].

At present, the use of CSF markers as a screening tool and enrichment criterion is feasible and recommended, due to the availability of recent development of reference standard procedures and materials. As compared to amyloid PET imaging, CSF markers are less costly and have a comparable accuracy. In

Table 1
Clinical trials in AD using CSF biomarkers for population enrichment (results from searching on clinicaltrials.gov)

Drug	Population	Trial phase	Definition of CSF biomarkers in the inclusion criteria	References
JNJ-54861911	Prodromal AD	I	Participants must have evidence of amyloid deposition as demonstrated by low CSF $A\beta_{42}$ levels at screening	NCT01978548 NCT02360657 NCT02406027
JNJ-54861911	Early AD	II	Participants must have evidence of amyloid pathology by means of either: a) low CSF $A\beta_{42}$ levels at screening; b) a positive amyloid PET scan at screening (depending on the site's PET capability) by visual read	NCT02260674
JNJ-54861911	Early AD	II-III	Participants 60 to 64 years of age must also have 1 of the following 3 conditions: a) a positive family history for dementia (minimum of 1 first degree relative), b) a previously known APOE ε4 genotype, c) a previously known biomarker status demonstrating elevated amyloid accumulation in CSF or PET	NCT02569398
Valaciclovir	Early AD	II	Diagnosed with AD or MCI due to AD. At least one brain imaging examination should have been done (CT, MR, SPECT, or PET/CT) and at least one objective finding should support the diagnosis beyond specific medical history. Reduced perfusion or reduced metabolism bilaterally temporally, hippocampal atrophy or pathological markers for AD in cerebrospinal fluid is such findings	NCT02997982
Elenbecestat (E2609)	Early AD	I	Meets the current cognitive classification of MCI or mild dementia due to AD pathology (all subjects having a "positive" biomarker for Aβ) as defined by the NIA-AA research criteria	NCT01600859
Elenbecestat (E2609)	Early AD	III	Positive biomarker for brain amyloid pathology as indicated by either amyloid PET or CSF assessment or both	NCT02956486 NCT03036280
Genistein	AD	III	CSF levels of Aβ, p-Tau compatible with AD.	NCT01982578
Exendin-4	Early AD	II	CSF $A\beta_{42}$<192 ($\pm$10%) pg/mL (given an intra-subject laboratory variability $\sim$ 10%)	NCT01255163
Lanabecestat	Early AD	II-III	For a diagnosis of mild AD, participant meets the NIA-AA criteria for probable AD. For a diagnosis of MCI due to AD, participant meets NIA-AA criteria for MCI due to AD	NCT02245737
Lanabecestat	Early AD	III	For a diagnosis of mild AD, participant meets the NIA-AA criteria for probable AD. For a diagnosis of MCI due to AD, participant meets NIA-AA criteria for MCI due to AD	NCT02972658
Lanabecestat	Mild AD	III	Meet the NIA-AA criteria for probable AD.	NCT02783573
LM11A-31-BHS	Mild to moderate AD	I-II	CSF AD specific biomarker profile; positive, defined as CSF $A\beta_{42}$<530 pg/mL together with either of t-Tau>350 pg/mL or p-tau >60 ng/mL	NCT03069014
BMS-241027	Mild AD	I	CSF consistent with AD pathology	NCT01492374
Nilotinib	Mild to moderate AD	II	Biomarker confirmed AD with CSF level of $A\beta_{42}$<600 pg/mL	NCT02947893
Avagacestat (BMS-708163)	Prodromal AD	II	CSF $A\beta_{42}$ levels < 200 pg/mL or Total Tau/$A\beta_{42}$ ratio of $\geq$ 0.39	NCT00890890

(*Continued*)

Table 1
(*Continued*)

Drug	Population	Trial phase	Definition of CSF biomarkers in the inclusion criteria	References
Solanezumab (LY2062430)	Prodromal AD	III	PET scan or CSF result at screening consistent with the presence of amyloid pathology	NCT02760602
Solanezumab (LY2062430)	Mild AD	III	PET scan or CSF result at screening consistent with the presence of amyloid pathology	NCT01900665
Gantenerumab (RO4909832)	Mild AD	III	CSF results consistent with the presence of amyloid pathology	NCT02051608

AD, Alzheimer's disease; MCI, mild cognitive impairment; NINCDS-ADRDA, National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association; NIA-AA, National Institute on Aging and the Alzheimer's Association; APOE ε4, apolipoprotein E, ε4 allele genotype.

the near future, we need to establish standard cutoffs to be used as inclusion criteria. In order to implement an effective strategy, we should handle the issue of between-site variability for CSF biomarker measurements. We also need to consider if previously measured CSF biomarkers might be considered as valid for inclusion.

DATA SHARING AND INDIVIDUAL PATIENT DATA META-ANALYSES

There is high interest among researchers in sharing data and protocols. Such an option allows the scientific community to comprehensively reanalyze previously collected data, encourage new interpretations, and promote research collaborations as well as enhanced transparency.

The use of electronic data capture methods consistently simplifies the task of data collection and has the potential to standardize many aspects of data sharing.

A trend toward increased sharing of neuroimaging data has emerged in recent years [31], and the CSF markers research field should follow the same path. Besides clinical trials, as a methodological approach in clinical research setting, data harmonization according to international standard formats should be constantly applied in order to make these data available to the scientific community, as in the ADNI experience (http://adni.loni.usc.edu).

Another aspect related to the sharing of raw-data is the conduct of individual patient data (IPD) meta-analysis, which is the gold standard for summing-up evidence. Despite the increasing availability of studies addressing many clinical issues, an intensive use of IPD meta-analyses is lacking. The IPD meta-analysis of Jansen and colleagues [32] provided interesting results suggesting a 20- to 30-year interval between the first sign of amyloid positivity and the onset of dementia.

CONCLUSIONS

CSF biomarkers entered the diagnostic criteria for AD. However, there are some steps to take in order to fully implement the use of CSF biomarkers in the AD routine diagnostic work-up and as strategy for enriching trial populations. We need to increase the general awareness of the importance of early diagnosis, the collaboration within the scientific community by promoting data sharing practices, and to encourage IPD meta-analyses.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9910).

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A Blood Test for Alzheimer's Disease: Progress, Challenges, and Recommendations

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Abstract. Ever since the discovery of *APOE* ε4 around 25 years ago, researchers have been excited about the potential of a blood test for Alzheimer's disease (AD). Since then researchers have looked for genetic, protein, metabolite, and/or gene expression markers of AD and related phenotypes. However, no blood test for AD is yet being used in the clinical setting. We first review the trends and challenges in AD blood biomarker research, before giving our personal recommendations to help researchers overcome these challenges. While some degree of consistency and replication has been seen across independent studies, several high-profile studies have seemingly failed to replicate. Partly due to academic incentives, there is a reluctance in the field to report predictive ability, to publish negative findings, and to independently replicate the work of others. If this can be addressed, then we will know sooner whether a blood test for AD or related phenotypes with clinical utility can be developed.

Keywords: Alzheimer's disease, blood proteins, blood tests, cohort studies, data reporting, genetics, gene expression, metabolomics, research design

PROGRESS

The identification of genetic markers such as *APOE* ε4 arguably represented the first step change in progress toward a blood test for late onset Alzheimer's disease (AD), as genetic markers can be measured from blood samples [1]. Since then, 19 other significant markers of AD have been identified by a genome-wide association study (GWAS) [2]. These markers have been combined into a polygenic risk score with ∼80,000 more-weakly associated genetic markers achieving an area under the curve (AUC) of 78% for prediction of AD. This compares with 72% achievable with just age, sex, and *APOE* ε4 (the 'co-variate only' model) [3]. In a smaller recent study (n ∼ 1,600), some of the same authors have shown that the same risk score has an AUC of 84% for predicting pathologically confirmed cases [4], which if confirmed in larger studies may have enough clinical utility to justify the use of genome-wide genotyping in the clinic to aid diagnosis. Even an AUC of 78%, if validated further, may have some utility for recruitment of higher risk individuals to prevention

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trials [5]. Other promising approaches at an earlier stage of development have involved increasing the GWAS sample size using an AD-by-proxy phenotype [6], and developing polygenic hazard scores to predict age of dementia onset [7].

AD polygenic risk scores have also been shown to be associated with brain atrophy [8, 9] and cerebrospinal fluid (CSF) amyloid-β [7, 8], but to the best of our knowledge they have not yet been shown to be predictive of these phenotypes. In fact, two independent studies ($n = 657$ and $n = 242$) have shown that this genetic risk score does not appear to be predictive of amyloid or tau pathology measured from CSF [10, 11]. A preliminary report that the polygenic hazard score is better able to predict elevated brain amyloid does not appear to test whether it improves upon a model using age, gender, and APOE alone, so its clinical utility is uncertain [12]. If the negative findings are correct, then this would be consistent with the idea that biomarkers must be optimized to specific use contexts, e.g., useful markers of AD diagnosis may differ from markers of pathology or progression. For this reason, the literature has broadened out from the case-control design to encompass endophenotype designs which look for markers of brain atrophy [13], CSF pathology [14], or cognitive decline [15]. However, it should be noted these findings do not yet seem to have translated into accurate prediction models [16].

Following on from the early genetic work, proteomic researchers joined the search for blood biomarkers of AD. In a clear parallel to earlier genetics research, two main approaches were taken: 'candidate' studies and 'discovery' studies. The AlzBiomarker project has performed a meta-analysis of association studies of 'candidate' blood (and CSF) markers with AD, showing that of all candidates in blood, total-tau appears to be the most associated with AD [17]. Predictive results from the use of blood total-tau are only provided for individual small-scale studies, so the reported sensitivity and specificity for predicting AD diagnosis is likely to be overly optimistic, e.g., 97% specificity and 91% sensitivity in Chiu et al. [18].

We have reviewed the 21 'discovery' blood protein studies published between 2002–2014, using a wide-range of proteomics techniques and AD-related outcomes. A low consistency of biomarkers identified between studies was observed, but four candidate biomarkers were observed in studies utilizing five independent research cohorts: α-1-antitrypsin, α-2-macroglobulin, apolipoprotein E, and complement C3. When examined in a new dataset, these proteins, when combined with age, sex, and presence of *APOE* ε4, had an AUC of 82% for predicting AD diagnosis, versus 79% for the co-variate only model [19]. While superficially better than the genetic risk scores AUC (78% versus 82%), it should be noted that the genetic result is more trustworthy as it comes from a much larger study and shows a greater difference in predictive ability between co-variate only and biomarker models.

In another parallel to genetic research, proteomic studies have also looked-for biomarkers of endophenotypes of AD, which have been reviewed in Baird et al. [20] who see promise in this area, but acknowledge limited success in identification of a reproducible signature. More recently, Nakamura et al., [21] have developed a blood test based on fragments of the protein amyloid beta that achieved an AUC of 94% for the prediction of elevated brain amyloid in a moderately sized study ($N = 232$). If shown to be robust, reliable, practical and affordable this could be a major breakthrough.

Blood metabolite studies of AD are more novel, with high profile papers by Mapstone et al. [22] and Proitsi et al. [23]. Mapstone et al. [24] identified ten lipids which could predict conversion from mild cognitive impairment to AD over 2-3 years with an AUC of 92%. Proitsi et al. [23] identified 24 metabolites which had an AUC of 71% for predicting AD diagnosis, in a considerably larger study ($n = 277$ versus $n = 85$).

Gene expression has also been explored as a potential source of blood biomarkers for AD. Little consistency has been seen in the genes selected by these various studies, leading Han et al. [24] to suggest that greater concordance might be seen at the pathway level. We demonstrated in Voyle et al. [25] a failure to replicate classifiers between independent sample sets, and that simple pathway level summaries of gene expression are no more predictive of AD. Endophenotype approaches have been explored in gene expression studies as well; for example Lunnon et al. [26] show that gene expression is predictive of brain atrophy. However, this work has not yet been replicated in independent cohorts.

More recently, we have been attempting to combine different modalities of biomarker to improve predictive ability. In Voyle et al. [27] we found that five metabolites could be used to predict amyloid positive individuals with 72% accuracy, rising to 79% when combined with levels of the protein fibrinogen gamma. This study was limited in size and requires

replication in independent samples. In a similar vein, we showed a marginal improvement in the prediction of CSF amyloid-β levels using genetic risk from the large AD genome-wide association study [2, 3] and plasma tau levels (AUC 67% versus 66% for covariate only model) [10]. Such a small improvement may be artefactual, and even if true is not likely to be useful by itself. Studies seeking to find multi-modal AD blood biomarkers and/or biomarkers of endophenotypes are likely to become more common but have been held back by the sample sizes available, which is a focus for improvement going forward.

CHALLENGES AND RECOMMENDATIONS

Further progress toward a clinically useful blood test will be slow unless we acknowledge and learn from the limitations of our current approaches; therefore what follows is our personal view on key challenges and important recommendations for future AD blood biomarker research. We draw attention to limitations of existing studies not to dismiss them, but to point out room for improvement in the field and in our own research. What follows will seem obvious to some, but needs to be highlighted as a counter-point to the over-optimism of the field.

The quality of experimental design in this field is variable, although this has been improving over time. One of the most obvious aspects of this is in sample size of non-genetic AD biomarker discovery studies, for example a study seeking to find blood protein AD biomarkers in 2002 used only 18 research participants [28]. Ten years later Doecke et al. [29] achieved $n \sim 1000$. This seemed a positive trend, but unfortunately, we are not aware of any larger studies published in the five years that followed. In fact, many smaller-scale studies ($n \sim 100$) are still published, e.g., [22, 27, 30, 31]. We should learn from the field of genetics, where small-scale candidate gene studies were plagued with replication issues [32] that were only solved by larger sample size and unbiased approaches. We recommend that this is tackled, in part, using samples from larger cohorts, such as UK Biobank [33] and the Precision Medicine Initiative [34], as well as cohort consortia such as the European Medical Information Framework – AD (http://www.emif.eu/) and Dementia Platform UK (http://www.dementiasplatform.uk).

Additional design considerations involve the appropriateness of the population used, and this should be guided by the anticipated context of use of the potential blood tests. Most studies have sought to find a blood test that could be helpful in the diagnosis of AD; however, none have yet been performed in the primary healthcare population in which it would have greatest utility [35]. This has been in large part due to the challenges of recruiting research participants, and the priority given to large scale recruitment rather than to representativeness of populations relative to anticipated context-of-use. Similarly, studies have sought to find AD markers that could be used to identify asymptomatic patients with early signs of AD, but very few have been performed in that population [36–39]. Another problem of unrepresentative sample populations is that they may not reflect the prevalence of AD related phenotypes (e.g., amyloid positivity) in populations appropriate to the anticipated context-of-use, which could inflate the positive and negative predictive values.

Partly due to the history of this field, which was initially led by clinicians and laboratory biologists, the level of statistical rigor is understandably variable. One example is the focus of many papers on *p*-values instead of predictive ability (sensitivity/specificity/positive predictive value, etc.). Significant *p*-values, even if replicable, do not necessarily mean that a biomarker is useful for predicting AD. To do so requires a suitably large effect size and a good understanding of confounding factors (e.g., age, gender, *APOE* ε4, medication use). For readers who may struggle to interpret and understand predictive measures, we heartily recommend Tze-Wey Loong's excellent visual explanation [40].

A critically important consideration is cross-validation, i.e., the assessment of predictive models in additional data not used in its construction. The data used in model construction is referred to as the training or in-sample dataset, whereas the independent data used for assessment is called the test or extra-sample data. It is important that predictive performance is reported from the test data, as results from training data can be artefactually better due to overfitting to noise [41]. This is equivalent in importance to blinding in clinical trials in the sense that it helps to protect results from the preconceptions of the researcher. This can be a major concern in studies which report predictive accuracy in training sets only, i.e., where no cross-validation has been performed. In some studies, k-fold cross-validation has been performed, in which the training data is repeatedly split into different training and test subsets and average performance in the test sets given. This is better than no cross-validation, but can still give overly gener-

ous predictive performance due to the train and test datasets sharing the same systematic noise, especially in small datasets and when $k < 10$ [42].

Despite seeming to perform cross-validation, researchers can often subconsciously and artefactually inflate predictive performance. The two most common examples of this are: 1) when both training and test data are used for variable selection, e.g., when variables are selected for model inclusion by ranking the *p*-values from repeated univariate tests using all available data (and therefore not correctly holding out test data); and 2) by performing correct cross-validation, finding a poor result which does not get reported, and then trying a new model (new variables, or new modeling approach or formula), only reporting models which have good predictive performance. The former mistake is one that we have made ourselves in Kiddle et al. [30]; the latter mistake is probably the most common which is evidenced in part by the relative absence of negative results published in this area. The absence of negative results is a form of reporting bias, leading to an overly optimistic impression of this field in the literature. While no evidence of reporting bias for 'candidate' blood protein markers of AD is seen in the AlzBiomarker meta-analysis, in the context of discovery and replication of blood biomarker panels, we are only aware of the following relevant papers showing negative results, some emerging from our own group [10, 25], some consistent with our negative findings [11], and others showing limited [43, 44] or no replication [45] of previous high-profile positive results [22, 46] when examined by independent researchers. It seems implausible that negative results are truly rare in AD blood biomarker research.

In terms of root causes, we are generally disincentivized to share negative or disappointing results, even if they are correct. However, the examples provided above demonstrate that it is simply not true that journals refuse to publish negative results, although we have certainly argued against peer reviewers who fail to see the value in doing so. In a worst-case scenario, negative results can be published on pre-print servers such as bioRxiv (http://biorxiv.org/). It may be worthwhile for the community to come together to generate a pre-registration platform, and to persuade journals to only publish pre-registered AD biomarker studies, so that groups failing to publish negative findings can be pressured into doing so. While not explicitly focused on blood markers, a very positive move in this direction has been challenges in which predictions are submitted before test data is released, notably the AD DREAM challenge [15], which generated a negative result, and the TADPOLE challenge which only recently stopped accepting submissions (https://tadpole.grand-challenge.org/).

Another cause could be confirmation bias, the psychological phenomena where individuals seek out evidence that matches their pre-conceived ideas [47]. This can mean that if a researcher believes a blood marker of AD will be found, they may disregard evidence to the contrary. This can lead to Hypothesizing After Results Are Known, researchers testing many different hypothesis (or models) reporting only the positive results and ignoring the multiple testing problem relating to all the unreported negative results [48]. This has also been called the file drawer effect, meaning that many published results are false positives [49]. John Ioannidis has discussed this in his controversially titled paper "Why most published research findings are false" [50]. While this focuses on *p*-values, it is likely to apply to studies reporting predictive performance as well. Interestingly, the field of blood biomarkers for AD fits his criteria for risk of high false positive publication rate: 1) small studies, 2) small effect sizes, 3) greater number and lesser pre-selection of tested relationships, 4) flexibility in designs and analyses, 5) financial interests and prejudice, and 6) many teams involved in a scientific field in chase of statistical significance.

The testing of promising biomarkers by independent researchers is essential to progress them toward the clinic [35]; however, this is rare. This has therefore been a focus of our research [19, 51]. Where this has been done for two high-profile studies [22, 46], they show a complete failure to replicate [45] or a significantly less promising performance [43, 44]. Less promising replications fit with the findings of John Ioannidis [52] who has studied the reasons that discovery results are typically inflated. In terms of clinical utility, a high predictive performance is required, meaning that partial replications in well-designed studies almost always rule out the clinical utility of the marker.

Given all the above, it is healthy to be skeptical about the potential of biomarkers in the current literature. It is a safe bet to assume that existing AD biomarker candidates at the very least require further validation and at worst are non-replicable, or are not of sufficiently high performance for clinical use. We hope this changes soon, but we believe that our recommendations may help the field to achieve this sooner.

While it is tempting to think that these problems may plague all blood biomarker research, this can be disproved by the example of blood protein markers of aging. Using proteomics techniques that have also been used in AD research, we have discovered plasma proteins correlating highly with chronological age, that replicate strongly in an independent cohort [53], in another cohort in serum [51], and in at least two studies by independent researchers [52, 53]. This shows that failures to replicate AD biomarkers cannot solely be blamed on the measurement technologies used, but it is certainly true that if a novel technology was able to detect a stronger AD related signal, then it is more likely to be replicated.

Given the large reproducibility crisis in science [50], and in this field specifically, how do we improve the way we do research to increase the chance that a reproducible blood test can be found? John Ioannidis has provided general recommendations for researchers to improve the chance of true positive findings that we think are relevant: "large-scale collaborative research, replication culture, registration, sharing, reproducibility practices, better statistical methods, standardization of definitions and analyses, more appropriate (usually more stringent) statistical thresholds, and improvement in study design standards, peer review, reporting and dissemination of research, and training of the scientific workforce" [56]. Other recommendations from John Ioannidis regard institutional changes affecting the incentives for scientists which would be even harder to achieve.

As highlighted by John Ionnadis, sharing and quality of reporting is important, and for that reason we strongly recommend that the field adopt the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) reporting guidelines [57]. Specifically for the reporting of pre-analytical variables, we recommend the advice provided by O'Bryant et al. [58].

In terms of sharing, we recommend that raw data should be shared as widely as possible, allowing independent statisticians to verify reproducibility of findings. This has been most successfully achieved through the Alzheimer's Disease Neuroimaging Initiative (https://ida.loni.usc.edu/), but is also being done to some extent by other cohorts including the Australian Imaging and Behaviour Longitudinal study of aging (https://ida.loni.usc.edu/) and AddNeuroMed (https://www.synapse.org/!Synapse:syn4907804). Clinicians leading large data collection need to be won over by the benefits of data sharing, including by inclusion of appropriate authorships (e.g., of clinical consortia), to counter the recent high-profile accusation that data sharing leads to "research parasites" [59]. While data sharing is done to some extent, the field would be greatly improved by researchers also sharing analysis scripts wherever possible. This would allow errors to be spotted, would make reproducibility straightforward, and would greatly assist junior researchers to develop the coding skills that are increasingly important for modern biomedical research. Code sharing can be facilitated by websites such as GitHub (https://github.com/), which allow both private and public sharing of code, as well as version control. Care must of course be taken not to release data that is sensitive, including within the script itself, but we have shown that this can be achieved [60].

Our final recommendation is that the ultimate aim is prediction models useful in a given clinical context, and that we should not be limiting ourselves to looking for markers in blood. Other variables, derived from routine clinical data such as electronic health records, wearables, or cognitive tests may have more promise for this purpose. This is not to say analysis of these datasets is not without its own challenges. Electronic health records represent sparse, incomplete, and often subjective representations of the disease state with important data often buried within free text narrative. The opportunities here are vast though, and although the datasets represent secondary use data, the data themselves are much larger and more representative of clinical settings than we are typically used to in blood biomarker studies, albeit less controlled in terms of co-variates and missing data. We have established research programs in this area and through information and extraction toolkits such as Clinical Records Interactive Search [61], CogStack [62], and the KConnect program (KConnect.eu), rolled out to multiple hospitals. We have used natural language processing to explore questions that include characterizing trajectories of cognitive decline with a specific focus on identifying and validating associations, with medications for example [63]. The ubiquitous use of smartphones and wearables devices provides the opportunity for a more objective, continuous, and pervasive phenotype, throughout the disease continuum from at risk, early diagnosis through to post diagnosis engagement, compliance and self-management. Such data provides the opportunity to augment our blood biomarker studies and clinical trials. We have established programs such as the RADAR-CNS (RADAR-CNS.org), a major goal of which is to develop a generalized real-time stream-

ing platform that will enable active (e.g., through questionnaires) and passive (e.g., accelerometry and heart rate using sensors on wearables and devices) remote monitoring, tracking phenotypes such as function and cognition.

CONCLUSIONS

The field of AD blood biomarkers has expanded to include genetic, protein, metabolite, and gene expression markers, as well as combinations of the above. While some consistency has been seen across independent studies, several high-profile studies have seemingly failed to replicate. At the same time, there appears to be a strong reporting bias for studies seeking to find a biomarker panel with few negative results published, making the true state of play impossible to assess. Will a clinically useful blood test for AD be developed? It is simply too early to say, but we will have a better chance if we can improve the design, analysis and reporting of studies. Many alternative markers exist within health records, from wearables or innovative cognitive tests, and these should also be explored.

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Potential Novel Approaches to Understand the Pathogenesis and Treat Alzheimer's Disease

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Abstract. There is growing genetic and proteomic data highlighting the complexity of Alzheimer's disease (AD) pathogenesis. Greater use of unbiased "omics" approaches is being increasingly recognized as essential for the future development of effective AD research, that need to better reflect the multiple distinct pathway abnormalities that can drive AD pathology. The track record of success in AD clinical trials thus far has been very poor. In part, this high failure rate has been related to the premature translation of highly successful results in animal models that mirror only limited aspects of AD pathology to humans. We highlight our recent efforts to increase use of human tissue to gain a better understanding of the AD pathogenesis subtype variety and to develop several distinct therapeutic approaches tailored to address this diversity. These therapeutic approaches include the blocking of the Aβ/apoE interaction, stimulation of innate immunity, and the simultaneous blocking of Aβ/tau oligomer toxicity. We believe that future successful therapeutic approaches will need to be combined to better reflect the complexity of the abnormal pathways triggered in AD pathogenesis.

Keywords: Apolipoprotein E, chronic traumatic encephalopathy, immunomodulation, innate immunity, oligomer, prion, Toll-like receptor 9, unbiased proteomics

INTRODUCTION

Alzheimer's disease (AD) is a complex, multifactorial disease, which is unique to humans. AD is defined neuropathologically by the accumulation of amyloid-β (Aβ) into extracellular plaques in the brain parenchyma and in the vasculature (known as congophilic amyloid angiopathy [CAA]), and abnormally phosphorylated tau that accumulates intraneuronally forming neurofibrillary tangles (NFTs) [1–4]. Pathological aggregation of phosphorylated tau and Aβ occurs in a sequential process. Monomers first aggregate into oligomers intraneuronally, which then continue to aggregate into the fibrils observed in amyloid plaques and NFTs, with this pathology then spreading in a characteristic brain topography that is distinct for NFTs and plaques [1, 5–7]. Much evidence indicates that oligomers are the most neurotoxic species in AD as levels of these species correlate much better with cognitive decline compared to the burden of plaques or NFTs [5, 8, 9].

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Amyloid plaques primarily consist of aggregated Aβ, with the most abundant forms of Aβ being $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$. However, amyloid deposits also contain Aβ species with heterogeneity at both the amino and carboxyl termini and with post-translation modifications such as pyroglutamate modifications at residues 3 or 11 ($A\beta_{N3pE}$ or $A\beta_{N11pE}$), as well as phosphorylation at serine residues 8 and 26 (pSer8Aβ and pSer26Aβ) [5, 10]. The presence and amount of these different Aβ species is important since some species are particularly prone to aggregation and are more toxic than others, with the presence of species such as pSer8Aβ having been linked to a "biochemical staging" of amyloid plaques [10, 11]. Evidence indicates this process initially occurs predominately in synapses [8, 12]. All species of Aβ are derived from cleavage products of the amyloid-β protein precursor (AβPP), a type 1 transmembrane protein present in all cells including neurons. In the amyloidogenic pathway, AβPP is initially cleaved by BACE1 and then cleaved by γ-secretase (a protease composed of presenilin-1, presenilin enhancer 2, nicastrin and APH-1), to release monomeric soluble Aβ (sAβ) [13], which has normal physiological functions with neurotrophic properties [14–16]. In AD, either increased production of sAβ and/or production of more aggregation prone species of sAβ (in the case of familial AD) or impaired clearance of sAβ (in the case of sporadic AD [sAD]) results in Aβ accumulation in the brain [5]. There are many environmental and genetic factors that increase the risk for AD; however, understanding the interplay between these risk factors and their individual contribution to the pathogenesis of AD, as well as in different subtypes of AD is a process in evolution. AD is characterized as either familial early-onset (EOAD; <5% of all AD patients, with onset at <65 years) or sporadic late-onset (sAD; onset >65 years). Autosomal dominant mutations in presenilin 1 (*PSEN1*), presenilin 2 (*PSEN2*), or the amyloid precursor protein (*APP*) gene account for ∼10% of all EOAD cases (∼1% of all AD cases), leaving the cause of the majority of EOAD unexplained [17–20]. sAD afflicts >95% of patients with AD and is related to both genetic and environmental factors [18, 21–23]. A combination of genome-wide association studies, linkage, and whole genome/exome sequencing have identified over 30 loci that confer increased risk for sAD, including genes involved in innate immunity, cholesterol metabolism, and synaptic/neuronal membrane function, suggesting that the pathogenesis of sAD has considerable heterogeneity [18, 20, 24, 25]. The strongest identified genetic risk factor for sAD is the inheritance of the apolipoprotein (apo) E4 allele, the protein product of which influences both the aggregation and clearance of brain Aβ [26–28]. Much more rare variants of another gene that encodes the triggering receptor expressed on myeloid cells 2 (TREM2) have been reported as a significant risk factor for sAD, with an odds ratio similar to apoE4 [29, 30]. This genetic diversity that drives AD pathogenesis suggests that AD is a syndrome with a final common pathway that involves the accumulation of Aβ and tau oligomers. Our understanding of these complex pathways has greatly increased in recent years; however, despite this expanding knowledge base there has been a very high failure rate of ∼99.6% with AD targeting clinical trials. There are many reasons for this high failure rate; however, an important factor has been the frequent premature translation of successful pathology reduction in transgenic (Tg) mouse models, which have pathology driven by overexpression of very rare EOAD mutations, to humans with sAD in whom the pathology is driven by substantially different pathways, which may vary in importance from patient to patient [3, 31–33]. In addition, studies in these animal models of AD ignore the very significant age associated neuronal loss that occurs in many brain regions, without correlation to NFT or Aβ pathology, that underlies an individual's "neuronal reserve" [34]. To overcome these limitations, one possible direction is greater research use of human tissue. AD pathogenesis heterogeneity could be better examined using omics approaches that allow genome- or proteome-wide screening for altered networks during disease, focusing on particular subset samples of AD [3, 35]. The use of various unbiased omics approaches is being increasingly recognized as essential for the future of effective AD research [36]. AD therapeutic approaches need to better reflect the diversity of disordered pathways that can drive AD pathology and be less amyloidocentric. In this review, we outline our recent attempts to use proteomic approaches to better understanding the heterogeneity of AD pathogenesis and our preclinical studies using a number of different, potentially synergistic, therapeutic approaches that we hope will have relevance for sAD.

PROTEOMIC STUDIES USING HUMAN POSTMORTEM TISSUE

There is a plethora of molecular alterations in the AD brain that occur in addition to the accumulation of pathologic Aβ and tau species. Proteomics offers

an unbiased comprehensive way to explore how these molecular changes are interrelated and how they contribute to AD pathophysiology. The hypothesis-free, exploratory nature of unbiased proteomics enables the simultaneous examination of thousands of proteins, which will ultimately provide a much broader overview of AD pathophysiology [37, 38]. The majority of proteomics studies using AD tissue have analyzed homogenates of large regions of tissue [39–50]. This approach has allowed the identification of proteins with large, region-wide differences between AD and controls. Many of these studies are limited by small numbers of subjects included and by the lack of sub-fractionation of tissue homogenates, meaning that detected protein changes are limited to abundant proteins only. However, these limitations are being addressed in more recent studies which include larger numbers of subjects across a range of disease states (ranging from preclinical to advanced AD), therefore providing an invaluable resource comprehensively detailing the proteome of the frontal cortex in these subjects [47, 51]. Other studies have used subcellular fractionation to examine protein alterations in AD in more targeted biochemically extracted fractions such as insoluble proteins [51–53], synaptic fractions [54, 55], phosphopeptides [56], and membrane associated proteins [57]. This type of approach allows more specific examination of proteomic differences in AD tissue in each of these fractions, but it still does not allow for direct analysis of proteomic differences in specific cell types or neuropathological features. Therefore, we developed our localized proteomics technique, which combines laser capture microdissection and label-free quantitative LC-MS to allow proteomics of specific regions, neuropathological features, or cells of interest [58–60]. Other groups have also used localized proteomics on frozen postmortem AD tissue specimens, analyzing the proteome of microdissected neurons [61], NFTs [62–64], plaques [65], CAA [66, 67], and specific hippocampal subregions [68]. However, a particular advantage of our localized proteomics technique is that it was deliberately optimized to allow the use of formalin-fixed paraffin embedded (FFPE) tissue. This is because the vast majority of human tissue specimens are FFPE blocks collected at autopsy, which are an underutilized, but exceptionally valuable resource for medical research.

We recently used localized proteomics to show that the protein composition of amyloid plaques was significantly different in patients separated into two subtypes of AD based on the rate of disease progression: those with rapidly progressive AD (rpAD) and those with typical sporadic AD (sAD) [35, 38, 69]. Patients with rpAD have a particularly aggressive form of AD where median survival time is limited to 7–10 months after diagnosis in comparison to a survival time of $\geq$10 years in sAD [70, 71]. Little is known about the pathological changes that underlie the rapid disease progression, and there are currently no gross neuropathological differences or differences in AD CSF biomarkers that can be used for either diagnosis or to explain the rapid progression of AD in these patients in comparison to sAD [70, 71]. However, it is important to note that recent studies have shown that $A\beta_{42}$ oligomers in rpAD have distinct properties from those in sAD, which may promote the faster spread of Aβ pathology [70, 72, 73]. The aim of our study was to compare the plaque proteome in rpAD and sAD patients ($n = 22$/group). We found that rpAD plaques had a significantly different protein composition in comparison to sAD; 141 proteins had significantly different levels in rpAD plaques [35, 38, 69]. Many of the proteins with altered expression are known to have a role in the development and maintenance of amyloid plaques (e.g., Aβ, gelsolin, GFAP, and α-synuclein), suggesting that these proteins may have a particularly important role in rpAD pathogenesis. Interestingly, rpAD plaques were found to contain significantly higher levels of neuronal proteins and significantly lower levels of astrocyte proteins. Immunohistochemistry validated and extended the proteomic data to show that the decreased levels of astrocyte proteins in rpAD plaques was due to fewer plaque-associated astrocytes in rpAD in comparison to sAD. Comparison of our plaque proteomic data with previous proteomic datasets generated using AD tissue showed that proteins with higher expression in rpAD plaques typically have either lower expression in sAD (39% of proteins upregulated in rpAD plaques are typically downregulated in sAD) or have no known involvement in sAD (46% upregulated proteins). In sum, this suggests that rpAD is unlikely to be simply a more extreme version of sAD, but instead a separate subtype of AD that is mediated by different pathological mechanisms.

One of the advantages of using unbiased proteomics to characterize protein differences is that it allows the detection and quantification of novel proteins linked to AD pathogenesis. One example of such a novel protein that we characterized in further studies is secernin-1. Very little is known about the general function of secernin-1, and its role

in AD has never been examined. Our proteomics data showed that secernin-1 had a consistently high expression in plaques in both rpAD and sAD [35]. Consequent immunohistochemistry showed that particularly high levels of secernin-1 were observed in plaque-associated dystrophic neurites and in NFTs. The consistently high degree of colocalization with phosphorylated tau could imply an important relationship between secernin-1 and the generation of NFTs. Further studies characterizing the distribution and function of secernin-1 in AD are currently underway. The detection of novel proteins involved in AD pathogenesis (such as secernin-1) highlights the powerful nature of unbiased 'omics studies. Studies such as these have great potential to increase our understanding of the broad molecular mechanisms that underlie AD and the large amount of data generated in these studies can be used as the basis for future targeted studies to specifically examine the role of each of these proteins in the development of AD [36, 38, 69]. The simultaneous analysis of thousands of proteins at once provides a much more complete overview of the molecular changes that occur in the AD brain and will ultimately help with the identification of the most promising drug targets beyond Aβ and tau.

APOLIPOPROTEIN E TARGETING THERAPEUTIC APPROACHES FOR AD

The apolipoprotein E (apoE)/Aβ interaction plays a major part in the conformational transformation of soluble Aβ and Aβ deposits in typical sAD (an exception is the subtype of rpAD, as discussed above) [26, 28, 70, 74, 75]. ApoE has a number of important functions in the brain, including being the major CNS cholesterol and other lipid carrier. It is also involved in synaptic plasticity, glucose metabolism, mitochondrial function, and vascular integrity [27, 28]. ApoE affects both the clearance and aggregation state of Aβ in an isotype specific manner in AD [26, 28, 75–77]. For example, apoE has been shown to enhance aggregation of Aβ with the order of apoE4 >apoE3 >apoE2 [78–81]; also, effects have been shown on the stabilization of Aβ oligomers, where apoE4 is found to have the greatest impact [82, 83]. Under physiological conditions, it has been determined that relatively little normal, sAβ binds to apoE [84] and apoJ is the major CNS Aβ binding protein [85, 86]. In AD, however, as the aggregate state of Aβ shifts, there is a greater interaction with apoE [77, 87, 88]. Research has also established that apoE4 is less effective at clearance of Aβ than apoE3 [89]. It is, therefore, possible to suggest that blocking the binding between apoE and Aβ could promote Aβ deposition, as it would inhibit clearance. However, pivotal *in vivo* studies show that this does not occur. Eliminating apoE reduces fibrillar amyloid deposition significantly [90], with apoE4 expressing AD Tg mice having greater amyloid deposition compared to apoE3 or E2 expressing mice [91, 92]. Further, other Aβ binding proteins, including apoJ or α2-macroglobulin, are associated with pathways that have greater effectiveness at Aβ clearance in contrast to apoE mediated Aβ clearance [26, 76, 77, 93]. It can be concluded, therefore, that the net effect of blocking the Aβ/apoE interaction is to inhibit deposition and enhance clearance. This strategy also has the advantage of not interfering with the many normal and beneficial functions of apoE. We have shown in several past studies, that treatment with Aβ 12-28P—a peptide homologous to the specific apoE binding domain of Aβ—in two AD Tg mouse models with primarily amyloid plaque deposition and in one AD model with primarily CAA, all produced a major reduction of Aβ burden, both in brain parenchyma and in brain vasculature when compared to age-matched vehicle-treated Tg mice [94–96]. Our studies additionally showed that blocking the apoE/Aβ interaction with Aβ12-28P in triple transgenic mice reduces AD-related Aβ and tau pathology [97]. In other results, Aβ12-28P treatment in an amyloid mouse model with apoE2-targeted replacement (TR) or apoE4-TR mouse backgrounds produced a reduction in Aβ oligomer and plaque load, also alleviating neuritic degeneration, which indicates that inhibition of Aβ/apoE interactions appears to materially block aggregation and deposition of Aβ, irrespective of apoE isoform [98]. In recent work, we sought to sharpen our approach toward possible clinical application. For this purpose, we undertook to design 9 pairs of related linear and cyclic peptoid compounds derived from the Aβ12-28P sequence to screen for new apoE/Aβ binding inhibitors, looking to demonstrate higher efficacy and safety [99]. The lead peptoid screened by surface plasmon resonance (SPR), CPO_Aβ17-21P decreased the apoE4/$A\beta_{42}$ binding at a 2:1 molar ratio (peptoid:apoE4) and virtually blocked all binding at a 8:1 molar ratio (peptoid:apoE4). The half-maximal inhibition (IC_{50}) derived from a

one-site competition, nonlinear, regression equation of CPO_Aβ17-21P was 1.02 nM, which is much improved compared to 36.7 nM for the parent peptide Aβ12-28P [95]. Other earlier studies showed that there is a critical region for Aβ binding to apoE in the residue range 17–21, with the lysine at residue 16 being special of importance [100, 101]. It can be expected that a peptoid conforming to this sequence would be a most effective inhibitor of the Aβ/apoE interaction. APP/PS1 AD mice treated with CPO_ Aβ17-21P had a major cognitive improvement, including reduction of soluble and insoluble Aβ peptide/oligomer levels in brain and lower total amyloid burden in cortex and hippocampus [99]. It is important to note that CPO_ Aβ17–21P treatment reduces Aβ related pathology and cognitive deficit using a 7.5 fold reduced dose (0.2 mg per mouse, twice per week) in contrast to the Aβ12-28P treatment dose used previously on 3xTg-mice (1 mg per mice, three times per week) [97, 99]. It suggested that the new peptoid inhibitor CPO_Aβ17–21P, with a very low molecular weight (<1 kDa) and inherent protease resistance, has improved bioavailability/biostability over Aβ12-28P.

There is a potential risk in targeting Aβ deposition in that increasing the pool of soluble Aβ may facilitate formation of the toxic oligomer species. That has been demonstrated by some other immunotherapeutic approaches [102, 103]. Although apoE has a dual role in Aβ deposition and clearance, CPO_Aβ17-21P inhibition of apoE4/$A\beta_{42}$ interaction in APP/PS1 AD mice did not affect the soluble Aβ pool. Another potential risk is brain inflammation when targeting Aβ deposition. Our work has shown, that Iba1 and CD11b (both markers for microgliosis), and GFAP (a biomarker for astrogliosis) immunoreactivity is reduced or unchanged in the CPO_Aβ17-21P treated Tg mice [99]. Our novel therapy of blocking apoE/Aβ interaction has ameliorated all AD pathological features tested, including: improved memory deficits, reduction of amyloid burden and tau pathology and reduction of vascular amyloid deposition [94–96, 99]. A research project utilizing Aβ12-28P to block apoE/Aβ interaction in an amyloid mouse model with apoE2-TR or apoE4-TR mouse background produced a reduction in Aβ plaque load and oligomer and ameliorated neuritic degeneration [98]. Therefore, it can be stated that this therapy is not apoE isoform restrictive. This approach does not preclude the simultaneous therapies discussed below, as they may have a synergistic effect that procures a more effective treatment.

STIMULATION OF INNATE IMMUNITY AS A THERAPEUTIC APPROACH FOR AD

Genome-wide association and other genetic studies have shown the linkage of a number of innate immunity related genes in late-onset AD, in particular TREM2 [29, 30, 104]. These studies are suggestive of the importance of microglia in AD pathogenesis, by identifying several AD associated genes that are expressed primarily in microglial cells. Microglia are critical regulators of innate immune responses in the brain. However, depending on the circumstances, their activation can have opposing effects [30, 105, 106]. Stimulation of innate immunity via Toll-like receptor (TLR) signaling pathways has been shown to be beneficial in modulating AD pathology in a number of studies [107–110]. On the other hand, manipulation of TLRs can also produce adverse effects in AD models [110–113]. Discrepancies between studies may be the consequence of variations in the types and doses of TLR ligands used, as well as administration frequencies. It appears that therapeutic immune activation should follow the "Goldilocks Principle": it needs to be just right. In addition, disease stage and underlying brain's immune status should be considered in designing future applications. We have focused on ameliorating immunosenescence and its associated AD pathology via TLR9 stimulation. TLR9 recognizes the unmethylated CpG sequences present at high frequency in bacterial and viral DNA and at low frequency in human DNA. Oligodeoxynucleotides (ODNs) containing these unmethylated CpG sequences trigger cells that express TLR9 (including cells of the monocytes/macrophage lineage, plasmacytoid dendritic cells and B cells) to mount an innate immune response. Several CpG ODNs have shown excellent safety profiles with >600 preclinical studies investigating the treatment or prevention of cancers, infections, and allergies, and >100 human clinical trials having been completed or are ongoing using CpG ODNs [114–117]. Immunotherapy has emerged as an attractive approach for disease intervention in AD; yet significant associated adverse events are the occurrence of amyloid related imaging abnormalities (ARIA) and cerebral hemorrhages, which are linked with the rapid clearance of CAA, with resulted blood-brain barrier break down, and excessive neuroinflammation [103, 118]. Our earlier studies using the Tg2576 and 3xTg-AD mouse models document that stimulation of innate immunity via

TLR9 ligand class B CpG ODN has the advantage of concurrently ameliorating Aβ and tau pathologies, in association with behavioral improvements [119, 120]. However, several studies suggest that inflammation and altered microglial activation may exacerbate tau deposition [121–123]. Our research findings clearly demonstrate CpG ODN reduces both tau and plaque pathology in 3xTg-mice [120]; however, in this study we could not exclude the possibility that the tau pathology reduction was secondary to the decreased amyloid burden. To resolve whether CpG ODN directly reduces pathological tau, we are currently conducting studies in Tg4510 AD model mice which have only tau related pathology.

The experimental mouse models utilized in our initial studies have minimal vascular amyloid. Hence, more recently we evaluated the therapeutic profile of CpG ODN in TgSwDI mice, which are an AD model with very extensive vascular amyloid [124, 125], testing the hypothesis that CpG ODN can harness innate immunity to reduce the age-dependent accumulation of CAA pathology in both young mice (prior to the onset of pathology) and in aged mice (with established pathology) [126, 127]. Our data documents that peripheral administration of CpG ODN negated short term memory deficits assessed by novel object recognition test as well as, being effective at improving spatial and working memory evaluated using a radial arm maze in both young and old age cohorts of TgSwDI mice [126, 127]. Detailed neuropathological evaluation accompanied by quantitative image analyses demonstrated significant reductions in total amyloid burden in CpG ODN-treated Tg animals compared to Tg controls. Even though fibrillar deposits are less amenable to clearance, quantification of Thioflavine-S stained sections confirmed a significant reduction in fibrillar vascular amyloid burden without associated microhemorrhages in CpG ODN groups. Importantly, we did not detect any microhemorrhages in wild-type animals after CpG ODN administration thus providing additional evidence of the safety of our approach. These favorable histological findings were corroborated by measurements of Aβ levels in the brain homogenates, which revealed a significant decrease in the levels of total and soluble $A\beta_{40/42}$ fractions and Aβ oligomer levels in CpG ODN-treated TgSwDI mice. Peripheral administration of TLR9 agonist, class B CpG ODN, successfully triggered a targeted immune response polarizing macrophages/microglia toward beneficial states of activation with improved phagocytic function, resulting in restriction of AD pathology in the absence of apparent toxicity. Therefore, our recent findings together with prior studies, validate this novel concept of immunomodulation as a safe method to successfully prevent and ameliorate AD related pathologies, supporting the potential clinical applicability of CpG ODN [119, 120, 126, 127]. Studies are currently ongoing using a non-human primate model of AD, squirrel monkeys, which naturally develop extensive CAA as well as ARIA, making them a particularly appropriate AD model to test an immunomodulatory therapeutic approach [128–130].

THERAPEUTIC IMMUNOMODULATION TARGETING Aβ AND TAU OLIGOMER TOXICITY CONCURRENTLY

Soluble oligomeric forms of Aβ and tau, which could spread via a "prion-like" mechanism, are thought to be the key mediators of neuronal toxicity in AD [5, 131–136]. The change in conformation to oligomeric misfolded conformers presents the possibility of specific immunological recognition using either active or passive approaches [103, 118, 137, 138]. Initial trials of active vaccination in AD failed as a result of autoimmune toxicity from the use of self-immunogens, such as aggregated Aβ [103, 139]. Clinical trials of passive immunization have also produced disappointing results related to the targeting of both physiological and pathological forms of Aβ, without specific targeting of the most toxic oligomeric species [103, 118, 140, 141]. It is now recognized that the soluble toxic oligomeric forms of pathologic proteins or peptides might be more efficient immunologic targets for both active and passive immunization approaches. This realization has led to the production of a limited number of anti-conformation monoclonal antibodies and new formulation vaccines, as was previously reviewed [103, 118, 137, 142]. Two significant problems need to be addressed for therapeutic success in targeting toxic oligomeric structures. The first is the widespread use of primary structure self-antigens to determine the tertiary structure of the oligomeric immunogens used for active immunization and the production/selection of possible anti-conformation monoclonal antibodies, with the remaining possibility of cross-reactive autoimmune toxicity due to incomplete selectivity for the pathological conformation. The second is the restrictive specificity of the immunogen to a single or limited number of pathological conform-

ers [141]. To overcome these problems, we recently developed a methodology to produce anti-β-sheet secondary structure conformational monoclonal antibodies [137]. Our prior work using three different AD Tg mouse models, has shown that active immunization based on this approach produces a therapeutic polyclonal response which reduces all key neuropathological features of AD, including amyloid plaques, CAA, and tau-related pathology, in association with significant cognitive improvements [103, 118, 143–145]. Amyloid plaques and CAA were shown to be reduced in APP/PS1 (amyloid plaque model) and TgSwDI (CAA Tg model) model mice, respectively, while in 3xTg-mice (amyloid plaque and tau pathology model) p13Bri immunization led to reductions of both tau and Aβ fibrillar and oligomeric pathology [103, 118, 143–145]. Inoculation of p13Bri with Alum as an adjuvant in these three AD Tg models produced a systemic polyclonal response to pathologic/oligomeric forms of both Aβ and tau, as well as demonstrating cross-specificity to AD, prion disease, and Lewy body disease human brain tissue (and not control human tissue). These promising results led us to the production of hybridomas from which we could select monoclonal antibodies (mAbs) with potential diagnostic or therapeutic value, by their specific reactivity to β-sheet secondary structures found in unrelated primary sequences of pathologic conformers of diverse neurodegenerative disorders [137]. The β-sheet secondary structure of proteins can be derived from very diverse and unrelated primary sequences, but generally is dominant in the production of any pathologic misfolded proteins or peptides. For an immunogen we used a small 13 amino acids peptide of the carboxyl terminus of the very rare British amyloidosis (ABri), which is derived from an intronic DNA sequence expressed by a missense mutation and has no sequence homology to any other mammalian protein (including all other known amyloid proteins) [118, 143, 146, 147]. The peptide was subject to controlled polymerization by an extensive glutaraldehyde reaction to form immunogenic, covalently bound 10–100 kDa soluble and stable oligomers with high β-sheet secondary structure content (p13Bri) [143, 144]. p13Bri inoculation, with a suitable adjuvant, produced an array of antibodies to the non-self motif and the β-sheet secondary structure. Stable hybridomas were obtained, with cloned mAbs selected by the novel approach of specifically using as selector compounds, oligomeric conformers from different neurodegenerative disorders with the only commonality being the shared β-sheet secondary structure. Due to the novel method by which we generated our anti-β-sheet conformational mAbs and their poly-reactivity to toxic conformers found in most common neurodegenerative disorders, we believe our approach to be innovative and more likely to have therapeutic success in humans, compared to other existing oligomer targeting mAbs [138]. The potential advantages include: 1) a reduced risk of inducing auto-immune complications since the immunogen used has no primary structure homology to any human peptide/protein (with the exception of ABri found in the very rare patients with British amyloidosis); 2) selective targeting of the β-sheet secondary structure found in toxic oligomers; hence, avoiding interference with the multiple beneficial and physiological functions of soluble Aβ, tau, α-synuclein, and PrP; 3) diminished risk of producing ARIA like complications, related to the direct clearance of fibrillar Aβ vascular deposits, since mainly oligomeric forms of Aβ and tau are being targeted; 4) simultaneous targeting of Aβ, tau, and α-syn related pathologic conformers (that have the potential to cross seed each other), addressing the mixed pathologies found in the majority of neurodegenerative disease patients [148–152]; 5) negligible risk of increasing toxic oligomer species by the mobilization of fibrillary Aβ and tau species as has been shown to occur with some vaccination methods [102]; 6) potential therapeutic activity for prion diseases by interfering with the spread of PrP^{Res}. No other reported methodology for producing mAbs to oligomeric conformations, published so far, has this unique combination of properties. Therefore, we believe that our technological approach has the potential to develop tools for the detection, monitoring and treatment of multiple neurodegenerative disorders [137].

Another somewhat related therapeutic approach is the potential blocking of both Aβ and tau oligomer mediated toxicity by inhibiting/competing with their binding to the normal PrP^{C}, which acts as an oligomer receptor on the surface of neurons. The Strittmatter group has demonstrated that extracellular oligomeric Aβ binds PrP^{C} with high affinity, activating an intracellular signaling cascade coupled to the protein tyrosine kinase Fyn [153]. The ability of oligomeric Aβ to activate Fyn is dependent on the presence of PrP^{C} and requires mGluR5, suggesting that in AD, oligomeric Aβ triggers neuronal signal transduction from PrP^{C} to mGluR5 to Fyn kinase [154–156]. Fyn activation, in turn, hyperphosphorylates and mislocalizes tau in the dendritic spines, leading to

destabilized microtubules, and the production of NFTs which results in the cognitive impairment characteristic of AD patients [156, 157]. We have previously shown that anti-PrP mAbs such as 6D11 can ameliorate cognitive deficits in an AD mouse model with advanced disease by blocking oligomer mediated synaptic toxicity via inhibiting binding to PrP^{C}, without affecting the amyloid burden or altering Aβ oligomer levels [158]. This same anti-PrP mAb is an effective therapeutic agent in prion disease models, preventing PrP^{Sc} replication [142, 159, 160]. More recently, we have also shown that PrP^{C} expression is critical in mediating tau-related pathology and neuronal toxicity in the setting of traumatic brain injury (TBI) [161]. TBI and its associated chronic traumatic encephalopathy (CTE) is now recognized to be a tauopathy related to tau oligomer mediated toxicity [161–163]. Hence, we hypothesize that PrP^{C} is a receptor for Aβ and tau oligomers, as well as, PrP^{Sc}. Therefore, blocking the Aβ, PrP^{Sc}, and tau oligomer interactions with PrP^{C} may reduce the pathology and cognitive deficits associated with AD, prion disease, frontotemporal dementia, and CTE.

CONCLUSIONS

Our understanding of the pathogenesis of AD has increased exponentially in recent years. Despite this growing knowledge base, translating preclinical therapeutic successes from animal models to the clinic remains an elusive goal. We believe that future therapeutic approaches need to better reflect the diversity of disordered pathways that can drive AD pathology. These approaches need to be tested using human tissue and a diversity of animal models that better reflect the wide spectrum of AD pathogenesis. Multiple different therapeutic approaches must be developed targeting the numerous distinct pathways that can ultimately lead to Aβ oligomer accumulation and the triggering of tau pathology. These therapeutic approaches such as the blocking of Aβ/apoE interaction, stimulation of innate immunity, and the simultaneous immune interference of Aβ/tau oligomer toxicity can be individually tailored to each patient depending on what is the primary driver of their AD pathology and/or their stage in the disease. In addition, it may be necessary to combine therapeutic approaches and/or to develop multi-target-directed ligands [164], to better reflect the complexity of the abnormal pathways triggered in AD pathogenesis.

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Brain Inflammation Connects Cognitive and Non-Cognitive Symptoms in Alzheimer's Disease

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Abstract. Alzheimer's disease (AD) is the main form of dementia in the elderly and affects greater than 47 million people worldwide. Care for AD patients poses very significant personal and economic demands on individuals and society, and the situation is expected to get even more dramatic in the coming decades unless effective treatments are found to halt the progression of the disease. Although AD is most commonly regarded as a disease of the memory, the entire brain is eventually affected by neuronal dysfunction or neurodegeneration, which brings about a host of other behavioral disturbances. AD patients often present with apathy, depression, eating and sleeping disorders, aggressive behavior, and other non-cognitive symptoms, which deeply affect not only the patient but also the caregiver's health. These symptoms are usually associated with AD pathology but are often neglected as part of disease progression due to the early and profound impact of disease on memory centers such as the hippocampus and entorhinal cortex. Yet, a collection of findings offers biochemical insight into mechanisms underlying non-cognitive symptoms in AD, and indicate that, at the molecular level, such symptoms share common mechanisms. Here, we review evidence indicating mechanistic links between memory loss and non-cognitive symptoms of AD. We highlight the central role of the pro-inflammatory activity of microglia in behavioral alterations in AD patients and in experimental models of the disease. We suggest that a deeper understanding of non-cognitive symptoms of AD may illuminate a new beginning in AD research, offering a fresh approach to elucidate mechanisms involved in disease progression and potentially unveiling yet unexplored therapeutic targets.

Keywords: Alzheimer, depression, amyloid- β, inflammation, microglia, TNF-α

INTRODUCTION

Medical and technical advances in the past several decades have significantly improved health and life quality, leading to extended lifespan worldwide. Notably in developed countries, but also in countries experiencing accelerated development, this has resulted in a curious demographic phenomenon, with an inversion in orientation of the population pyramid and an increase in the proportion of older to younger individuals [1]. Although an extended lifespan remains a desirable goal, this also led to a concomitant increase in prevalence of age-related diseases, including various degenerative disorders such as cancer or dementia. Dementia represents the major cause of functional dependence in the elderly, currently affecting 47 million people worldwide and with an incidence of 9.9 million new cases per year [2]. Alzheimer's disease (AD) is the main cause of dementia, representing 60–80% of dementia cases in

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the USA [3]. It is estimated that US$ 818 billion are spent per year worldwide in treatment and care of AD patients [4]. It is, thus, clear that AD is a major and unmet economic and public health problem, and that there is an urgent need for development of effective therapies capable of preventing its progression or even reversing it.

Memory loss, the clinical symptom most commonly associated with AD, usually manifests first as an incapacity to form and store new memories, followed by progressive impairment in recalling older memories [5]. Brain regions related to memory are affected early in AD, but the progression of the neurodegenerative process gradually compromises regions related to other cognitive abilities, such as language and motor control [3]. Significantly, along with the classical and well-known cognitive impairment, AD patients may also experience important non-cognitive symptoms related to self-consciousness and emotionality, which further deteriorate quality of life, especially at early stages of the disease. These non-cognitive symptoms, often referred to as Behavioral and Psychological Symptoms of Dementia (BPSDs), include affective and psychological disturbances [6], the most prevalent ones being apathy and depression [7, 8]. Management of BPSDs is thought to represent one third of the costs involved in dementia care [9, 10]. BPSDs thus represent important co-morbidities in AD, from both individual and public health standpoints.

Although AD was first described 110 years ago [11], its mechanisms of pathogenesis have remained largely elusive. As a consequence, attempts to develop therapies for AD have mostly met with failure or, at best, with modest results. There are currently four drugs approved by the FDA for AD treatment, namely three cholinergic drugs (rivastigmine, donepezil, galantamine) and one NMDA receptor blocker (memantine), none of which are efficacious in terms of curing the disease or preventing its progression. Moreover, the scenario is not encouraging, as memantine, the last drug approved for AD treatment, has been on the market for more than 14 years. During this period, a large number of drugs have reached clinical trials but failed to reach pharmacy shelves. Thus, it is imperative that alternative approaches to counteract AD progression be investigated.

Notably, the vast majority of the therapeutic strategies examined to prevent AD progression so far have been based on memory loss-related mechanisms and showed very modest results in terms of both cognitive and non-cognitive symptoms [12]. Investigation of mechanisms implicated in non-cognitive symptoms of AD could unveil novel targets for therapeutic strategies. In this review, we focus on mechanisms underlying BPSDs and their impact on AD patients. A better description of molecular/cellular pathways involved in BPSDs appears warranted to allow a deeper understanding of AD pathogenesis, and to guide future advances toward disease-modifying drugs to prevent or treat this devastating disorder.

ALZHEIMER'S DISEASE, A DISEASE OF MEMORY

The original report by Alois Alzheimer in 1907 (commented in [11]) identified two distinct protein aggregates in histopathological analysis of an AD brain: intracellular neurofibrillary tangles (composed of tau protein in hyperphosphorylated form) and extracellular senile plaques, composed primarily of the Aβ peptide released into the brain parenchyma upon amyloidogenic processing of the amyloid-β protein precursor (AβPP). Aβ is physiologically produced by neurons, and at low concentrations (pM) appears to play important roles in synaptic plasticity and memory-related processes [13–20]. In AD, Aβ accumulates in the brain as a result of an imbalance between production and clearance [12], culminating in its aggregation to form plaques [21]. For many years, the amyloid cascade hypothesis staged senile plaques as the major cause of neurodegeneration and memory loss in AD [21].

Early studies, however, showed that there is no clear correlation between memory loss and plaque load in AD patients [22, 23]. Moreover, Aβ immunization approaches were found to have significant and fast beneficial effects on memory in transgenic mouse models of AD, under conditions in which total brain levels of Aβ or plaque load were not affected [24, 25]. These and other early studies challenged the notion that amyloid plaques were the central toxic species in AD.

Mounting evidence accumulated in the past two decades now indicates that soluble oligomers of Aβ (AβOs) are the main neurotoxins in AD [12, 26–28]. This view is supported by the ability of AβOs to bind specifically to neurons [29, 30], notably to excitatory dendritic spines [31], and to induce synapse damage/loss and memory impairment by multiple mechanisms, e.g., [32–40]. Therefore, recent efforts

have aimed to provide a better understanding of the deleterious roles of AβOs in the AD brain.

AβOs comprise a heterogeneous family of soluble assemblies, ranging from dimers/trimers to 24mers or even larger oligomers [26, 41]. Several lines of evidence indicate that distinct oligomer assemblies may have distinct deleterious actions on neurons. For example, dimers, trimers, and tetramers play critical roles in impairment of synaptic plasticity, likely attributable to synapse damage and loss, e.g., [37, 42–48]. On the other hand, high-n oligomers appear to exhibit preferential binding to synaptic sites [49], and to trigger N-methyl-D-aspartate (NMDA) receptor-mediated increases in intracellular calcium levels and neuronal oxidative stress, e.g., [37, 50, 51], among other deleterious mechanisms.

In addition to the fact that our understanding of the mechanisms triggered by AβOs is still incomplete, the identity of the synaptic proteins/receptors targeted by oligomers remains unclear. It is now becoming clear that AβOs present a promiscuous interactome, with more than 15 different neuronal proteins reported to bind oligomers [52]. These include, among others, NMDA receptors [50, 51], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR; 53), metabotropic glutamate receptor (mGluR5; [54–57]), neurexin/neuroligin [40, 58], and PrPc [59, 60] (reviewed in [28]). Given the multiplicity of proteins found to interact with AβOs, it seems plausible that oligomers recruit a multi-protein receptor cluster that mediates multiple signaling mechanisms culminating in synapse dysfunction [26, 28]. Thus, the heterogeneity of AβO assemblies [41], and the multiplicity of their binding partners at the neuronal surface and of mechanisms by which they impact synapse health contribute to enhance the complexity of AD pathogenesis.

Once bound to synaptic terminals, AβOs profoundly impact neuronal homeostasis. It is well established that AβOs impair long-term potentiation (LTP) in hippocampal slices and *in vivo* [32, 33, 61]. Early events triggered by AβOs include aberrant NMDAR-dependent calcium signaling, triggering excessive reactive oxygen species production in neurons [50]. This in itself is a likely mechanism underlying impaired synaptic plasticity [62] and, ultimately, leading to neurodegeneration. AβO-induced synaptic pathology is further associated to endocytosis of NMDA and AMPA receptors from the neural surface (e.g., [35]), and with aberrant activation or inhibition of pathways that interfere with normal memory processes, including the integrated stress response and translation (e.g., [36, 38, 61]). Disruption of synapse physiology by AβOs eventually leads to synapse loss (e.g., [37, 38, 40, 63, 64]), thus compromising neuronal activity and connectivity.

Notably, a single infusion of a low dose of AβOs into the lateral cerebral ventricle is sufficient to impair memory in mice [37, 38, 40, 64, 65]. Similarly, numerous transgenic mouse models of AD based on overproduction and brain accumulation of Aβ exhibit consistent cognitive deficits upon aging, and this correlates with impaired LTP and synapse loss (e.g., [61, 66, 67]). Identification of AβOs as synaptotoxins and the likely causative agents of memory/cognitive impairment in AD has stimulated efforts to neutralize their exacerbated activity in AD brains. Different anti-Aβ antibodies have reached clinical trials, but treatment of AD patients with these drugs led to generally disappointing outcomes. The most promising results have resulted from use of Aducanumab (Biogen), an Aβ antibody that recognizes both oligomers and fibrils, which afforded some delay in cognitive decline of AD patients [68]. The authors of the study suggest that diminishing plaque load, considered a likely source of soluble Aβ species, together with blocking AβO effects by Aducanumab may protect patients against cognitive decline. Despite critical evidence of the central role played by AβOs in the memory/cognitive facet of AD, their effects on non-cognitive symptoms of AD have remained much less clear.

ALZHEIMER'S DISEASE, A DISEASE OF MEMORY? NON-COGNITIVE, NON-TRIVIAL SYMPTOMS OF AD

Memory loss experienced by AD patients is often accompanied by BPSDs [7, 69], related to disturbed perception, thought content, mood, or behavior [70]. BPSDs include a variety of conditions, such as depression, apathy, anxiety, delusions, hallucinations, agitation, euphoria, irritability, aberrant motor behavior, aggression, wandering, and sleeping and eating disorders [7]. Although frequent and well described in AD, it is not clear whether BPSDs are consequences of disease progression, part of the physiopathology, or both. There are currently three main hypotheses to explain the development of BPSDs during the progression of AD.

The unmet needs model [71]. This model posits that dementia patients are not able to attend to specific needs, as they lose their communication and

self-providing abilities [71]. Most common unmet needs described by patients are loneliness and boredom. Curiously, specific unmet needs yield distinct anomalous behaviors, such as vocal or physical agitation [72]. The authors suggest that understanding the unmet needs of individual patients could be used to design nonpharmacological interventions to treat AD [73–75].

The symptom hypothesis [76]. This hypothesis suggests that the neuropsychiatric symptoms of AD are a reflection of the spread of pathology across different brain regions. In line with this hypothesis, the appearance of certain BPSDs earlier in life may predict future development of dementia [77]. Severity of depression, as well as other BPSDs, including apathy, have a positive correlation with disease progression, which can be explained by degeneration and synaptic failure of circuits controlling behavior [7, 78].

The risk factor hypothesis [79]. The risk factor hypothesis states that BPSDs are risk factors for AD establishment, rather than consequences of pathology. Indeed, studies from the past decade indicate that depression, for example, may not be an early symptom but rather a risk factor for dementia [80]. The Multi-Institutional Research in Alzheimer's Genetic Epidemiology (MIRAGE) study enrolling 4,046 subjects showed a significant association between history of depression and AD risk [81, 82], suggesting that depression could predispose to the development of AD.

Distinct BPSDs arise with degeneration and morphological alterations of specific brain structures and circuits [76]. For example, early symptoms such as irritability, hallucinations, and anxiety suggest degeneration of monoaminergic circuitry [83–85], which may also impact cognitive mechanisms. Indeed, Martorana and colleagues [84] reported that dopamine agonists were capable of restoring cognitive deficits in AD patients. Moreover, aggressive behavior in AD is associated with atrophy of frontolimbic structures [86]. Finally, depressive symptoms appear related to decreased gray matter volume in frontal and temporal lobes [87], lesioned subcortical regions [88], and reduced glucose metabolism/brain perfusion in the prefrontal cortex [89–94]. Therefore, the course of neurodegeneration in AD could determine the onset and development of BPSDs associated to pathology.

Interestingly, the occurrence of BPSDs not only correlates with brain degeneration but also with external factors, such as caregiving and environment [95]. Caregivers play a relevant role in AD progression. Programs designed to train caregivers in how to deal with demented patients have had positive results decreasing both caregiver burden and patient behavioral symptoms [95, 96]. On the other hand, wrong strategies implemented by caregivers negatively impact the occurrence of BPSDs in AD patients [97]. Moreover, a higher prevalence of stress and depression is observed in AD caregivers than in caregivers for non-demented patients [97]. Family members are the most affected, since 75% of AD patients are cared for by friends and family at home [98]. Therefore, cognitive and non-cognitive symptoms associated with AD directly impact mental health of both patients and caregivers.

Despite their obvious social and clinical relevance, and in contrast with the investigation of mechanisms underlying cognitive symptoms and memory loss, few studies to date have addressed molecular mechanisms underlying non-cognitive symptoms of AD. Animal models exhibiting non-cognitive symptoms of AD have been little explored in recent years (e.g., [65, 99–103]). Although relatively neglected for a long time, a better comprehension of the relation between disease progression and the development of BPSDs may shed light into mechanisms of AD pathogenesis and set the basis for a new beginning in AD investigation.

Aβ OLIGOMERS AS KEY PLAYERS IN NON-COGNITIVE SYMPTOMS IN AD

Similar to AD patients, transgenic murine models of AD present several BPSDs [65, 99, 104, 105]. These findings indicate that Aβ could mediate not only cognitive symptoms of AD, but also non-cognitive defects. Although this notion is well supported, it remains unclear whether AβOs directly bind to synapses subserving non-glutamatergic circuits, or whether their actions on other neuronal types rely on the propagation of pathology throughout brain circuitry. In the following sections, we discuss neuropathological mechanisms involved in three well described BPSDs, namely eating and sleep-wake disorders, which are related to hypothalamic dysfunction, and mood disorders, such as apathy and depression, related to monoaminergic circuitry deregulation.

Eating disorders

Although weight loss is a common feature in 20–30% of AD patients [106, 107], little is known about the underlying mechanisms. The increase in

food intake accompanying weight loss suggests a hypermetabolic state [108, 109]. Early postmortem analysis of AD brains identified Aβ deposits in the hypothalamus [110, 111] and a reduced hyphothalamic volume, together with a decrease in the number of orexigenic neurons [112, 113]. In line with these findings, PET scan analysis of AD brains revealed decreased hypothalamic volume and grey matter [114–116]. Intriguingly, a reduction in glucose metabolism in the hypothalamus precedes cognitive impairment in the Tg2576 transgenic mouse model of AD [117], in harmony with early hypothalamic degeneration in AD patients [118]. Thus, hypothalamic dysfunction offers an attractive explanation for eating disorders related to AD.

Interestingly, i.c.v. infusion of AβOs in mice and monkeys triggers insulin resistance in the hypothalamus [119]. In mice, this was accompanied by increased food intake [119], similar to what was previously described for AD patients [106]. Of note, Tg2576 mice present elevated orexigenic neuron activity [120], and AβO-infused mice presented increased levels of the orexigenic neuron protein marker, NPY [119], offering a possible explanation for altered food intake in AD. Furthermore, AβOs impair hypothalamic response to insulin, a known repressor of appetite [119]. These data suggest that AβOs orchestrate eating disorders in AD.

Although it is known that Aβ forms deposits in the hypothalamus and that soluble Aβ species could target hypothalamic neurons, the molecular mechanisms implicated in this pathology are still largely unknown. We showed that AβOs induce an increase in phosphorylation of eukaryotic initiation factor 2α (eIF2α-P) in the hypothalamus [119], similar to what takes place in the hippocampus [38, 61]. In both brain regions, this is mediated by the pro-inflammatory cytokine TNF-α, as infusion of Infliximab, a TNF-α neutralizing antibody, prevents eIF2α-P elevation. Increased levels of eIF2α-P attenuate overall protein synthesis, thus impacting memory processes in the hippocampus and possibly underlying metabolic deregulation and eating disorders in the hypothalamus.

Sleep disorders

Sleep disturbances are present in 25 to 90% of AD patients (reviewed in [121]), and include sleep fragmentation, excessive daytime napping, and decreased slow-wave sleep [122, 123]. These usually precede the onset of cognitive symptoms in AD [124]. The sleep state reached is crucial for consolidation of specific types of memories: while Rapid Eye Movement (REM) sleep is important for consolidation of non-declarative memories [125–127], non-REM sleep is essential for consolidation of declarative ones [128, 129]. Therefore, reduced sleep or an imbalance in sleep states could represent a risk for proper cognitive functionality.

Wakefulness increases Aβ levels, exacerbating and accelerating the onset of AD in animal models [121, 124]. On the other hand, increased Aβ levels cause a reduction in total sleep time, reducing both REM and N-REM sleep [130]. Levels of Aβ in cerebrospinal fluid (CSF) and interstitial fluid (ISF) appear to be upregulated by orexin, the hypocretin that regulates wakefulness [131, 132]. Indeed, knockout of orexin in APPswe/PS1ΔE9 mice, a transgenic mouse model of AD, markedly decreased Aβ levels in the brain, and at the same time increased total sleep time [132]. Furthermore, Tg2576 mice present amyloid plaque deposits in cholinergic areas that regulate sleep, such as mesopontine tegmentum [133, 134]. These findings are in harmony with an early report by Rudelli and colleagues [135], that identified amyloid deposits in cerebral cortex, basal forebrain, locus coeruleus, and hypothalamus in AD brains. In accord with observations in AD patients, Aβ-based transgenic models show early sleep disturbances, usually preceding cognitive impairment [130].

Antibody-mediated neutralization of Aβ in APPswe/PS1ΔE9 mice normalized sleep-wake cycle, indicating that Aβ plays a central role in deregulation of circadian cycles in murine models of AD [130]. Indeed, a single infusion of AβOs into the lateral cerebral ventricle was sufficient to induce a decrease in sleep time in mice [136]. Conversely, and demonstrating a bidirectional relationship between sleep and Aβ, chronic sleep restriction in mice led to increased sensitivity to the impact of AβOs on memory [136]. The synergism between chronic sleep restriction and AβOs in cognitive impairment appears to be mediated by TNF-α, as this effect was blocked by Infliximab [136]. This indicates that inflammation plays a pivotal role in the induction of sleep disorders by AβOs.

Mood disorders

Apathy and depression are prevalent BPSDs in AD. Nonetheless, epidemiological data on prevalence of apathy and depression in AD show high variability, depending of the studied cohort, method-

ology, and diagnostic criteria [137–141]. Although some aspects of the symptomatology of apathy and depression—including reduced interest, motivation and energy—overlap in definition [142], a differential diagnosis of depression involves the presence of sad mood, guilty feelings, low self-esteem, and hopelessness [8].

Kent et al. [143] were the first to link sickness behavior and cytokines in experimental animals. Since then, depression has been largely related to microglial dysfunction and secretion of pro-inflammatory cytokines [144–146]. The "inflammatory hypothesis" of AD [147–149] points to inflammation as a central player in AD pathogenesis, suggesting that microglial activation is not a consequence of neuronal dysfunction but rather a pathological feature related to disease development and progression.

Transgenic mouse models of AD, including APPswe/PS1ΔE9 and 3xTg mice, present depressive-like behavior [102, 104, 107] suggesting that increased Aβ levels may be responsible not only for cognitive symptoms, but also for the development of AD-associated mood disorders [150]. Moreover, we found that a single infusion of AβOs into the lateral cerebral ventricle is sufficient to induce despair behavior and anhedonia in mice [65, 102]. These effects were accompanied by increased levels of activated microglia in the hippocampus and cortex, and by elevated brain levels of two major pro-inflammatory cytokines, TNF-α and interleukin-1β [65]. Neutralization of TNF-α with Infliximab blocked the induction of depressive-like behavior in AβO-infused mice, thus establishing that brain inflammation mediates crucial events in AβO-triggered depressive-like behavior [102].

Serotonin (5-HT) depletion is largely associated with depressive behavior (reviewed in [151, 152]). This appears to be modulated by inflammation, as pro-inflammatory cytokines have been shown to enhance 5-HT turnover in the brain [153–157]. Moreover, reduced serotonin levels have been associated with AD pathology [17, 158]. Notably, mice infused via i.c.v. with AβOs present microglial-dependent reduction in brain serotonin levels [102]. Conversely, serotonin treatment alleviates inflammation and prevents the development of depressive-like behavior in AβO-infused mice [102]. Therefore, an interplay between serotonin and inflammation appears to play a crucial role in depressive-like behavior induced by AβOs in an AD mouse model.

Antidepressants have long been employed for treatment of depression in AD [159, 160], with results showing clear mood improvement in patients. Remarkably treatment with fluoxetine not only rescued depressive-like behavior but also memory deficits induced by AβOs in mice [65]. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI), and it was shown to be involved in stimulation of neurogenesis in the hippocampus [161, 162] and to have anti-inflammatory actions [163]. Indeed, fluoxetine greatly reduced microgliosis in the brains of mice infused with AβOs [65]. These results provide preclinical support to the use of an FDA-approved antidepressant as a therapeutic approach to combat memory loss in AD [164, 165].

MICROGLIA TAKES CENTER STAGE IN AD PATHOGENESIS

Results described above define an important role for AβO-induced brain inflammation in cognitive and non-cognitive symptoms of AD. In particular, TNF-α is increased in AD brains and in animal models of AD [65, 102, 166, 167], and blocking its activity by using neutralizing approaches protects mice from AβO-induced memory deficits and BPSDs [38, 102, 136].

For a long time, the brain was considered an immune-privileged organ [168]. Knowledge on the role of microglial cells in brain physiology was generally limited to a classical point of view, in which microglia were considered to be in a resting state until their activation by pathogens or toxic molecules [169]. It is clear now, though, that microglia are in constant surveillance of the brain milieu [170] and are responsible for multiple physiological processes, including neurogenesis and synaptic stability [171]. Similarly, knowledge on the role of microglia in disease, including AD, has increased significantly during the past decade. Originally staged in AD as clearance cells, which would be involved in plaque isolation [172] and in the removal of amyloid aggregates from the brain [148, 173], microglia are increasingly considered a central piece in the emergence and progression of AD (reviewed by [146]). Enlightening the role of microglia in AD could unveil alternative pathways for AD treatment.

Along this line, Krstic and Knuesel [147] proposed a new hypothesis for AD, based on the pro-inflammatory state characteristic of preclinical models and AD patients. This new *inflammatory hypothesis* points to microglial activity as a crucial piece in the progression of AD pathology. However,

it remains a matter of debate whether glial activation is cause or consequence of AD, or even a protective response [174]. Microglia present dichotomic activity and can switch between distinct activation states depending on environmental inputs. Microglial profiles in these two states are very similar to the extensively described M1 and M2 macrophagic profiles, in which M1 presents a pro-inflammatory bias, while M2 has anti-inflammatory activity [146, 175]. Fan et al. [176] showed that microglial activation changes from a neuroprotective anti-inflammatory M2-like to a neurotoxic pro-inflammatory M1-like state with the progression of AD, thus establishing a correlation between microglial profile and disease severity, as previously suggested by Sheng and collaborators [177]. Similar M1-M2 microglial dynamics was described by Jimenez et al. [178] in a transgenic mouse model of AD. Clinical and preclinical results thus suggest that the participation of microglia in AD is highly dynamic, varying during the disease course.

The role of microglia in the inflammatory process developed during AD progression has been studied during the past decade. Distinct transgenic mouse models of AD show increased microglial activation, which is accompanied by increased secretion of pro-inflammatory cytokines [102, 179, 180). Interestingly, prevention of microglial activation by minocyclin or microglial ablation using encapsulated sodium clodronate protected mice from AβO-induced depressive-like behavior [102]. Notably, modulation of microglia protects mice not only from non-cognitive symptoms but also from cognitive impairment induced by AβOs [181]. Thus, modulating microglial activation may favor protective mechanisms related to microglia/neuron interactions, which may—at least in part—explain the protection against cognitive and non-cognitive symptoms.

An inflexion point in consideration of the role of microglia in AD was determined by recent human genome wide association studies, which pointed out that polymorphisms in specific genes related to the immune system could be associated to elevated risks of AD development [182–184]. Among such genes, the one codifying Triggering Receptor Expressed on Myeloid cells 2 (TREM2) has attracted considerable interest [185]. TREM2 is a surface receptor present in microglial cells, regulating both phagocytic capacity and interaction with amyloid plaques [185]. Intriguingly, TREM2 ablation in different AD transgenic mouse models results in opposite phenotypes [186]. While TREM2 knockout in APPswe/PS1L166Pmice resulted in alleviation of pathology [187], in 5xFAD mice it boosted pathology [188]. The latter findings are consistent with reduced levels of secreted TREM2 in the CSF of AD patients [189]. These findings suggest that TREM2 has a central role in the pathology, although more studies are necessary in order to clarify its precise role.

The phagocytic activity of microglial cells has been implicated in decreased number of synapses in AD brains. The engulfment and phagocytosis of synapses (synaptic pruning) was recently demonstrated to be regulated by the complement system, soluble immunogenic molecules that associate with the plasma membrane in response to injury [190]. Although the complement system is extensively characterized in peripheral organs, it was only recently shown to mediate pro-inflammatory processes in the brain. In AD models, AβOs greatly increase brain levels of C3, one of the proteins in the complement cascade, which is tightly correlated to synapse loss [191]. Significantly, ablation of C3 in APPswe/PS1ΔE9 mice prevented cognitive impairments, despite a significant increase in total Aβ plaque density [192].

The precise mechanisms by which AβOs activate microglia are still unidentified. AβOs bind to microglial surface receptors and trigger signaling pathways, including Toll like receptors (TLRs) [193]. TLR4, which is highly expressed in microglial cells that surround Aβ plaques in AD brains [194, 195], appears crucial for AβO-mediated glial activation, since its ablation protected mice from AβO-induced depressive-like behavior [102].

The findings described above place microglia at the center of AD pathogenesis. According to this new view, AD should no longer be regarded a cell-autonomous neuronal disorder, but rather a disease that involves aberrant communication between neurons and microglia. This opens up a whole new area in AD research, and significantly increases therapeutic opportunities. Although ablation of microglial activity in the brain is possibly not viable, and probably not even desirable, we suggest that pharmacological rebalancing of microglial activity could offer an attractive, yet unexplored therapeutic strategy for AD.

CONCLUDING REMARKS

AD was long regarded essentially a disease of memory, as neuronal dysfunction and neurodegeneration impact memory-related brain structures early in

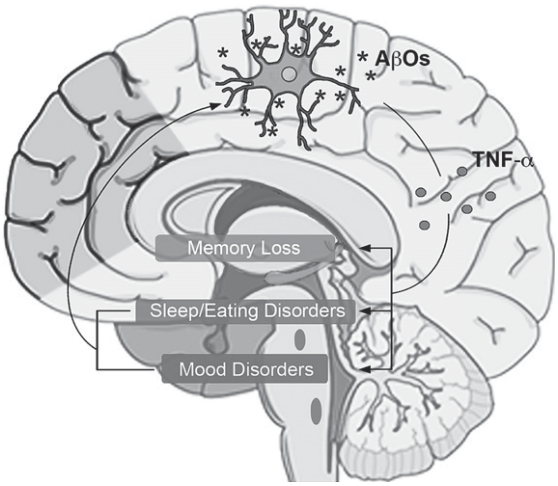

Fig. 1. Microglial activation links cognitive and non-cognitive symptoms of AD. According to the *risk factor hypothesis* (see text), non-cognitive behavioral alterations (such as eating/sleep disorders, and mood alterations) may increase the risk of AD development. This could be mediated by microglial activation, which results in increased secretion of pro-inflammatory cytokines, including TNF-α. On the other hand, perturbations in brain homeostasis leading to increased levels of AβOs amplify microglial activation to a pathological state. In line with the *symptom hypothesis* (see text), inflammation would drive neuronal dysfunction and neurodegeneration in different brain structures, triggering cognitive and non-cognitive symptoms of AD. Specific symptoms are related to dysfunction in distinct brain structures. Memory loss is closely related to hippocampal and frontal cortex dysfunction (blue), eating/sleep disorders are related to impacts in the hypothalamus (green), and mood disorders are connected to dysfunction in the striatum and raphe nuclei (red).

the disease process. Recent evidence, however, points to an important role of non-cognitive symptoms of the disease. The nature of these symptoms questions whether they are consequences of neurodegeneration spreading across brain regions, or early pathogenic events or risk factors that culminate in AD development. It is clear that a better understanding of AD demands a greater comprehension of BPSDs.

The role of microglia in the inflammatory component of AD pathogenesis offers a remarkable link between cognitive and non-cognitive symptoms of AD (Fig. 1). In fact, not only microglial cells but also astrocytes have attracted increasing interest in recent studies of AD pathogenesis (e.g., [64, 196, 197]). Studies to better elucidate the role played by each cell type in AD have evidenced intriguing facets of pathogenesis and have revealed alternative mechanisms that could translate into novel therapeutic approaches. Efforts should thus be aimed at understanding the role of glial cells in AD pathogenesis, in hopes that such knowledge may contribute to the development of efficacious approaches to block disease progression.

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Toward a New Concept of Alzheimer's Disease Models: A Perspective from Neuroinflammation

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Abstract. The continuing failure to develop an effective treatment for Alzheimer's disease urges a better understanding of the pathogenic mechanisms and the improvement of current animal models to facilitate success for clinical interventions. The transgenic models have been so far designed to recapitulate one, or both, protein lesions found in the brain of patients, the extracellular amyloid plaques and the intraneuronal neurofibrillary tangles. However, in recent years, a third pathogenic component is gaining strength in the onset and progression of this disease, the neuroinflammatory response mediated primarily by the brain's resident immune cells, microglia. This has been highlighted by the identification of genes involved in innate immunity as risk factors to develop this neurodegenerative disease. Our current concept, mostly derived from amyloid-β producing models which show a robust microglial activation, supports an initial beneficial role of these glial cells followed by a pro-inflammatory cytotoxic function later on. This view is now challenged by emerging data in human postmortem samples. We have recently demonstrated that in the hippocampus of Braak V-VI individuals there is a prominent degenerative process of the microglial population, driven by phospho-tau, that might compromise neuronal homeostasis. This scenario of microglial dysfunction/degeneration should be taken into account for developing more reliable animal models of this disease and improve their predictive value for human drug efficacy testing. Finally, correcting dysregulated brain inflammatory responses might be a promising avenue to restore cognitive function.

Keywords: Alzheimer's disease, degeneration, inflammatory, innate immunity, microglia, transgenic models

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Alzheimer's disease (AD) is the most common cause of dementia in people over the age of 65 leading to a high burden of care worldwide [1]. Histopathologically, AD is manifested by the presence of extracellular plaques consisting predominantly of amyloid-β (Aβ) peptides, and neurofibrillary tangles composed of intraneuronal aggregates of hyperphosphorylated tau protein [2]. Amyloid plaque

deposition begins 15–20 years prior to the onset of symptoms [3, 4], and after this long preclinical phase, tau pathology begins to spread from the entorhinal-hippocampal region to neocortical areas showing a good correlation with cognitive decline and brain atrophy [5]. Therefore, the amyloid cascade hypothesis posits that amyloid accumulation resulting from an altered balance between production and clearance of Aβ peptides is the seeding factor that triggers AD pathology and dementia [6]. However, so far targeting the amyloid cascade has failed to show efficacy in clinical trials [7–10] revealing a higher and still unsolved complexity for AD pathology. In the last few years, increasing evidence indicates that neuroinflammation involving particularly microglia and astrocytes contributes to AD pathogenesis and disease progression [11–15]. Thus, understanding and controlling the cerebral innate immune pathways may lead to new therapeutic opportunities for the prevention or delay of AD. Microglia, the primary cellular component of the brain's innate immune response, are complex and dynamic phagocytic cells that can have supportive or detrimental effects on neurons depending on their activation phenotype and secreted factors [16–19]. Upon activation, microglia also suffer morphological changes displaying larger cell body along with shorter, thicker, and less branched processes. In AD, activated microglia accumulate around Aβ plaques in both amyloidogenic transgenic models [20–22] and AD patients [23]. Although microglial activation may help to restrict amyloid pathology (by Aβ phagocytosis and/or plaque compaction) at early disease stages, microglial function might lose efficacy or even become detrimental, contributing to neurotoxicity later on [24]. The recent identification of several genetic risk factors involving proteins associated with microglial function highlights the role of these cells in AD pathogenesis (for review see [25–29]). In particular, a rare missense genetic variant (R47H) in the gene encoding the triggering receptor expressed on myeloid cells 2 (TREM2) significantly increases the risk of late-onset AD [30–32]. TREM2 which is expressed by microglia in the brain is a lipid sensor implicated in regulation of phagocytosis, inhibition of inflammatory signaling, and promotion of cell survival [33, 34]. In AD, TREM2 supports Aβ-reactive microgliosis and Aβ clearance [35]. In the absence of TREM2, microglial activation is impaired. In the 5xFAD model, TREM2 deficiency increased Aβ accumulation due to a dysfunctional microglial response, even more, microglia were apoptotic instead of activated [35]. This defect in the microglial barrier function around plaques led to less compact Aβ fibrils and a prominent axonal damage [36]. Decreased plaque-associated microgliosis was also observed in human R47H variant carriers as a result of TREM2 impairment [36]. Thus, a deficient neuroprotective microglial response rather than an overactive cytotoxic phenotype, could, indeed, be associated with AD development. In agreement with these data, we have recently reported microglial degeneration in the hippocampus of AD patients [37]. As discussed below in more detail, this novel scenario of microglial degeneration/dysfunction associated with AD pathogenesis (see also [38]) is completely opposite to the extensive microglial activation seen in the amyloidogenic AβPP-based mouse models. Therefore, microglial impairment should be reproduced in these animal models in order to improve their predictive value for treatment efficacy in humans. In this article, we summarize some of our findings to highlight the differences in the microglial response between AD models and human brains.

AMYLOID-DRIVEN MICROGLIAL ACTIVATION IN MOUSE MODELS AND HUMAN BRAINS

Data from transgenic animal models and human postmortem samples revealed that Aβ plaques cause an immune response in the brain. Microglia, as well as astrocytes, acquire an activated phenotype and cluster around fibrillar amyloid deposits. The current concept is that such an activation is primarily a protective response aimed at removing/isolating injurious stimuli by removing/compacting Aβ and creating a protective barrier. However, during the course of the disease and due to chronic activation microglia may lose this beneficial phenotype as they acquire a 'toxic' phenotype characterized by the production of pro-inflammatory mediators. However, most knowledge concerning the microglial response in AD has been obtained using Aβ producing models, such as AβPP-transgenic mice. These transgenic models displayed a strong response to extracellular Aβ accumulation [22]. In this sense, as we have previously shown in the $APP_{751SL}/PS1_{M146L}$ model [20, 21, 37], microglial activation could be easily identified by either molecular and morphological approaches. In fact, multiple different genes were transcriptionally affected by the microglial activation. As shown in Fig. 1A, the expression of surface or lysosomal markers (CD11b, Iba1, Cd45, TREM2,

and CD68) experienced a clear and highly significant increase in the AβPP-model hippocampus, as compared with age-matched WT mice. Activated microglial cells specifically surrounded Aβ plaques (Fig. 1C, D) and exhibited a typical "active" morphology, i.e., enlarged cell body with short and thick processes (Fig. 1C2, D1), clearly different from the "resting" non-activated microglial cells found in the inter-plaques areas (Fig. 1C1). These activated microglial cells that cluster around amyloid plaques adopted an alternative ''M2" phenotype identified by the expression of YM1 and the neurotrophic factor IGF1 [20]. In support of a protective microglia associated to plaques, recently, using single-cell RNA sequencing a novel microglial subtype (named DAM from disease-associated microglia) with the potential to restrict disease has been identified in an AβPP-based model (5xFAD) [39]. As the disease progresses with age, microglial activation also increases in response to a continuous Aβ plaques build-up in the brain (Fig. 2A1-3). Interestingly, in aged AβPP/PS1 mice activated microglial cells were also seen in the interplaque areas besides those around plaques. This interplaque activation was coincident with the accumulation of relatively large amounts of soluble oligomeric Aβ peptides (Fig. 2D and [20]).

Recently, we have also evaluated the microglial reaction in the hippocampus of human postmortem AD samples [37]; however, the results were somehow quite different to that in AD models. As shown (Fig. 1B), the expression of markers of microglial activation was slightly increased (i.e., CD45) or not affected in the Braak V-VI population (demented patients), as compared with non-demented Braak II individual, suggesting the absence of any strong microglial response as observed in the AβPP/PS1 mice. In the same way, using CD45 as a marker of active microglial cells (the expression of this protein was low in the resting or non-active microglia), we were able to detect few microglial cells at the hippocampal region, always surrounding Aβ plaques (Fig. 1E, E1). Therefore, in contrast with the large and significant microglial activation usually observed in the hippocampus of AβPP-based models, hippocampal human AD samples displayed a weak and limited microglial activation, restricted to amyloid plaques.

The different reported amyloidogenic mouse models remain largely consistent in their phenotypes with a robust microglial response (for review, see [22, 40]), and therefore all of them fail to recapitulate the weak nature of the inflammatory reaction of AD patients. This discrepancy between mice models and humans is further supported by the failure of numerous immunomodulatory compounds to display efficacy in treating the human disease despite success in preclinical animal models of AD [40–42].

DIFFERENTIAL AMYLOID AND PHOSPHO-TAU ACCUMULATION IN AD MODELS AND HUMAN BRAINS

The different microglial response described above between AD samples and transgenic models could in fact be explained by differences in the Aβ accumulation. As mentioned above, the hippocampus of AβPP-based models concentrates large amounts of Aβ plaques as disease progresses (Fig. 2A). The amount of Aβ that aggregates as extracellular deposits was clearly higher in aged transgenic mice than in human AD samples (compare Fig. 2A3, B). When directly compare, by western blots (using the same protein loading), the total Aβ extracted from models and AD brains, the transgenic AβPP/PS1 model produced by far larger quantities of amyloid than Braak V-VI demented samples (see Fig. 2D). However, both mice and human hippocampus presented a similar pattern of plaque distribution, with amyloid plaques accumulation mostly located at the perforant path afferent fields suggesting an axonal origin of the Aβ from synaptically connected regions. Most of the plaques were surrounded by AT8-positive dystrophic neurites (Fig. 2B1) and a halo of oligomeric Aβ (Fig. 2B2). The marked difference in the amyloid accumulation between AD cases and transgenic models, at least at the hippocampal formation, could in some degree explain the vast difference in the microglial response.

On the other hand, as previously reported [5, 43, 44], the hippocampal region is a phospho-tau enriched region. As shown in Fig. 2C, AT8-positive staining was highly found through the whole hippocampal formation; particularly in the stratum oriens of CA fields, enthorinal cortex, and subiculum and pre-subiculum, in the form of neurofibrillary tangles, neuropil threads, and dystrophic neurites (see Fig. 2C1). This robust phospho-tau accumulation could also be demonstrated by western blots. As shown (Fig. 2E), Braak V-VI samples accumulated large amounts of phosphorylated and aggregated AT8-positive tau. This accumulation was larger than that observed in AβPP-based models (not shown, see

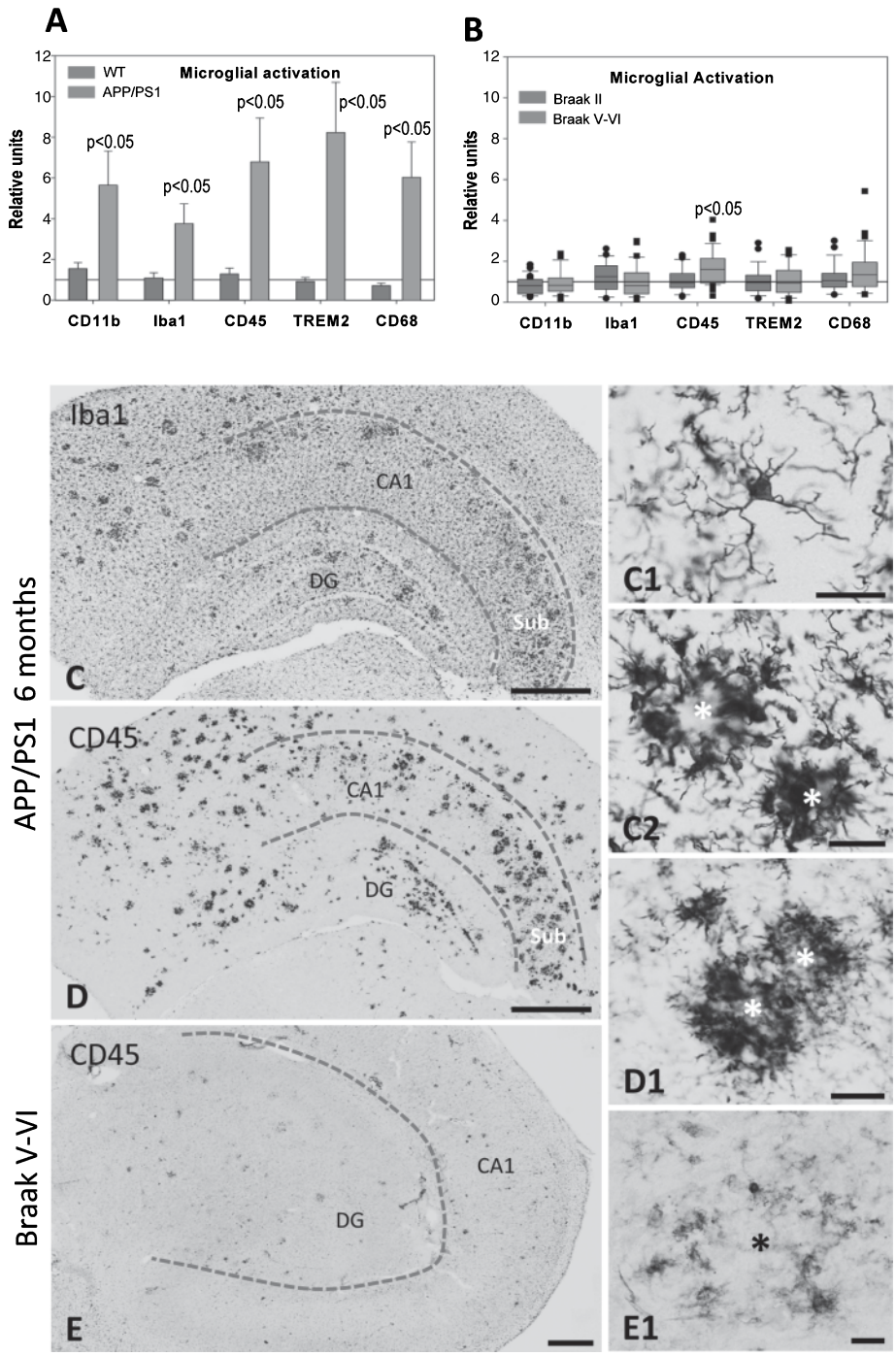

Fig. 1. Attenuated microglial activation in the hippocampus of AD brains (Braak V–VI) compared to AβPP/PS1 transgenic mice. Microglial activation was analyzed (qPCR) in 8–12-month-old AβPP/PS1 mice ($n = 10$ for each genotype) (A) and postmortem human samples with Braak II ($n = 21$) or Braak V–VI pathology ($n = 28$) (B). Expression levels, normalized using GAPDH, were referenced to WT mice or Braak II samples. Significance was analyzed by two-tailed *t*-test (for mice samples) or Mann Whitney test (for human samples). Immunostaining for Iba-1 (C) and CD45 (D and E) in the hippocampus of 6-month-old AβPP/PS1 mice (C and D) and postmortem human Braak V-VI samples (E). Higher magnification images show a non-activated Iba-1-positive microglia (C1), and activated Iba-1-positive (C2) or CD45-positive (D1) microglia that cluster around amyloid deposits (asterisks) from AβPP/PS1 hippocampus. E1) CD45-positive microglia located around an Aβ plaque (asterisk) from human hippocampus. CA1, hippocampal field; DG, dentate gyrus; Sub, subiculum. Scale bars: C, D, and E, 500 μm; C1, C2, D1, and E1, 20 μm.

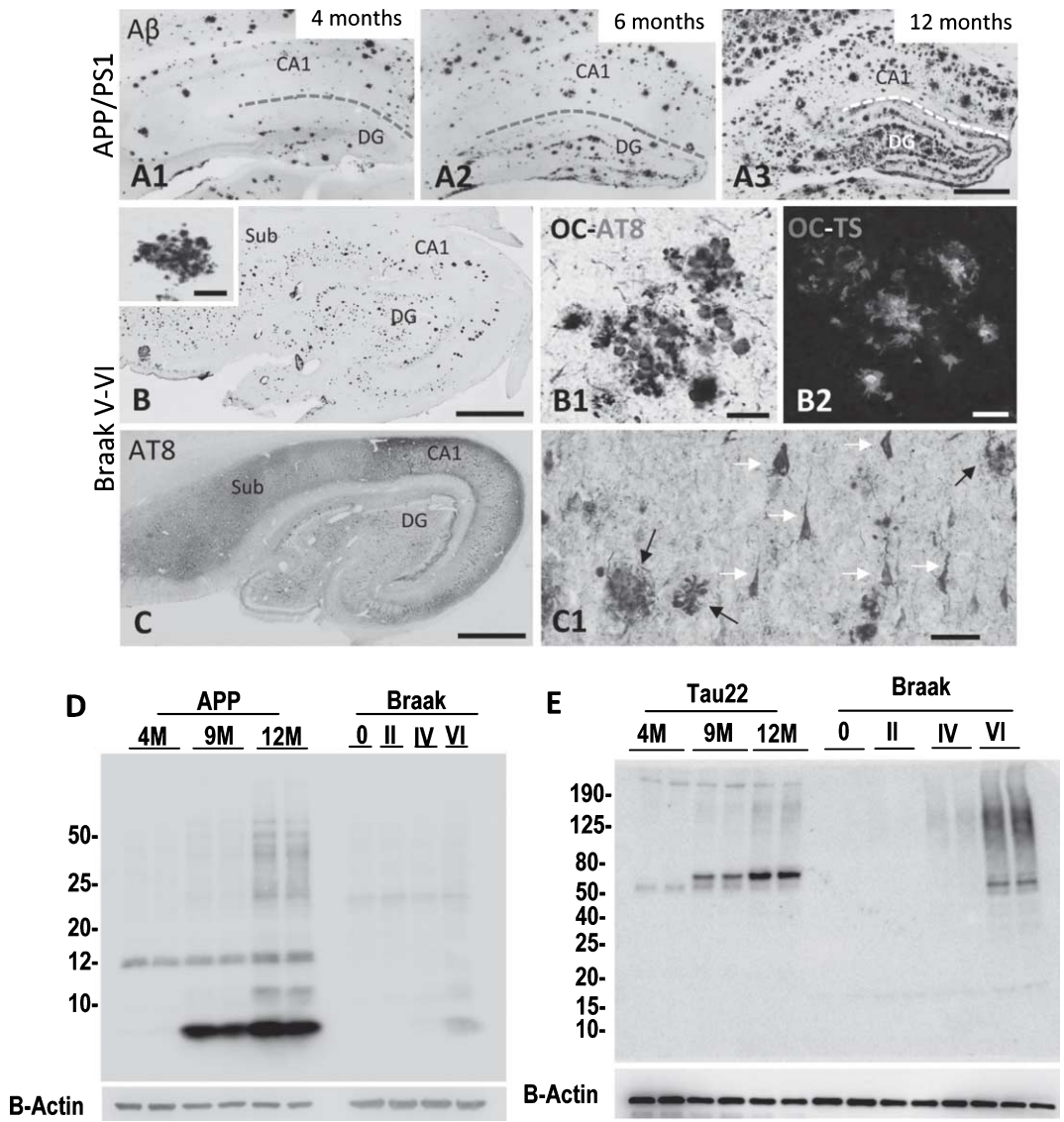

Fig. 2. Differential expression of Aβ and phospho-tau in the hippocampus of AD models and human brains. A1-3) Robust accumulation of Aβ plaques in the hippocampus of AβPP/PS1 mice with age as revealed by $A\beta_{42}$-immunostaining. B) Amyloid deposition in the hippocampus of Braak V-VI stage by $A\beta_{42}$ immunohistochemistry. B1) Double immunolabeling for Aβ (OC antibody; dark blue reaction) and phospho-tau (AT8 antibody; brown color reaction) revealing numerous AT8-positive dystrophic neurites around plaques. B2) double fluorescence labeling for Aβ (OC antibody; red labeling) and Thioflavin-S (fibrillary Aβ; green labeling) showing the presence of an oligomeric Aβ halo in the periphery of fibrillar plaques. C) AT8-immunostaining in the hippocampus of same Braak V-VI individual. C1) Higher magnification image of CA1 region showing neurofibrillary tangles (white arrows) and dystrophic neurites around plaques (black arrows). D) Western blots (10 μg of protein per sample), using 82E1+6E10, showing the extensive expression of monomeric/oligomeric Aβ species in the hippocampus AβPP mice (with a rapid increase from 4- to 12-month-old) compared to humans (from Braak 0 to VI stages; Braak VI are AD patients group). E) Western blots (10 μg of protein per sample), using AT8 antibody, demonstrate the differential expression of phospho-tau species in the hippocampus of Thy-Tau22 mice compared to humans with the highest expression of phospho-tau in the Braak VI samples. CA1, hippocampal field; DG, dentate gyrus; Sub, subiculum. Scale bars: A1-A3, 500 μm; B and C, 2 mm (inset in B, 50 μm); B1 and B2, 20 μm; C1, 50 μm.

[45]) or even in the transgenic tau model Thy-tau22 (Fig. 2E).

Therefore, amyloidogenic (expressing Aβ) and tau (expressing phospho-tau) models, or even the reported models with plaques and tangles (expressing both Aβ and phospho-tau), are not able to reproduce similar amounts of the pathogenic proteins as seen in human brains with sporadic AD. This incongruity

between mice and patients might be responsible for the distinct inflammatory response elicited by the pathogenic aggregated proteins.

MICROGLIAL CELLS DEGENERATE IN THE HIPPOCAMPUS OF AD PATIENTS

Keeping in mind the unequal Aβ and phospho-tau accumulation detected in transgenic mice and human brains, we have also analyzed the expression of microglial specific genes. In this sense, RNA-seq experiments using isolated microglia have clearly demonstrated that these cells express a subset of genes that constituted the "microglial signature" [39]. Within these specific genes, we have determined the expression of three of them, such as CX3CR1, P2ry12, and Tmem119 (see Fig. 3A, B). As shown, in the AβPP model, the expression of these particular genes (Fig. 3A) was either not altered (P2ry12, Tmem119) or exhibited a small but significant increase (CX3CR1) compared with age-matched WT mice. Since it was also known that the expression of these particular genes decreased upon "activation" [39], these data were somehow conflictive. In fact, our own *in vitro* experiments, using primary microglial cells, demonstrated that the expression of these microglial genes decrease after stimulation with either LPS or oligomeric Aβ (not shown). However, these results could also reflect the proliferation of microglial cells in these models [46]. In fact, we also observed an increase in the expression of the mitotic marker Ki67 (Fig. 3G) paralleled by increase in the BrdU incorporation on microglial cells [46]. Interestingly, the expression of the same microglial specific genes was significantly reduced in the hippocampus of human Braak V-VI samples (Fig. 3B). It could be argued that this decrease simply reflects the activate status of the microglial cells. However, as we pointed out above, the microglia were not activated in AD samples, at least by molecular and morphological evaluation (Fig. 1E). Therefore, the decrement in expression of these microglial genes may reflect a decrease in the microglial population. Indeed, a marked reduction in the Iba-1-positive microglial cell population was identified by immunostaining in the hippocampus of Braak V-VI individuals (Fig. 3D, D1) compared to Braak II cases (Fig. 3C, C1).

This reduction showed a regional pattern DG>C3>CA1>parahippocampal gyrus (see [37]). In this sense, we have demonstrated using Iba-1 as a marker, that microglia displayed a degenerative process in Braak V-VI samples. This process implicated a reduced number of cells, at least in 50% of cases, at the hilar region of the dentate gyrus as determined by stereology (Fig. 3F). Furthermore, as show in Fig. 3 (E3, E4), the microglial pathology was characterized by shortened and less branched processes that usually were deformed, displaying cytoplasmic abnormalities including spheroids and even fragmentation (cytorrhexis). Similar AD-associated microglial altered morphology has been reported by others [43, 47]. This degenerative phenotype of the microglia was easily distinguished from the non-activated (Fig. 3E2) or activated (Fig. 3E1) microglia. This microglial degenerative process produces a prominent decrease in the parenchymal area covered by microglia including Aβ plaques. On the other hand, we have also demonstrated that soluble phospho-tau was toxic for microglia. Also, this soluble phospho-tau was increased in Braak V-VI samples, as compared with Braak II [37]. Thus, the reduced number of microglia, and the degenerative status of the remaining, in AD samples could be due to a toxic effect of this soluble phospho-tau. In this sense, recently it has been published that microglia actively phagocytose soluble tau species [48]. Therefore, an increase on the levels of toxic soluble phospho-tau species together with the capacity of microglia to internalize these toxic proteins could produce the microglial degenerative process observed in AD samples.

However, as mentioned above, microglial cells could proliferate when activated by Aβ. Therefore, the toxic effect of soluble phospho-tau could be compensated by an increase in their proliferative capacity. We indirectly tested this point by measuring the expression of Ki67 in the postmortem human samples. As shown (Fig. 3G), we did not detect any significant modification in the expression of this proliferative marker between Braak II and Braak V-VI samples. Therefore, the proliferative capacity of the microglial cells was not increased in AD, in contrast to the clear effect on AβPP-models. In this sense, it is interesting to note that human microglial cells retained the proliferative capacity [49]. Furthermore, it is also noteworthy that, at least in mice models, the proliferative rate was particularly high in the dentate gyrus of the hippocampal formation [50]. Although the reasons for this differential rate were not known at present, it might indicate the existence of a high turnover of microglial cells in this particular hippocampal region, highly affected in our AD samples. Thus, it is tempting to speculate that the accumulation

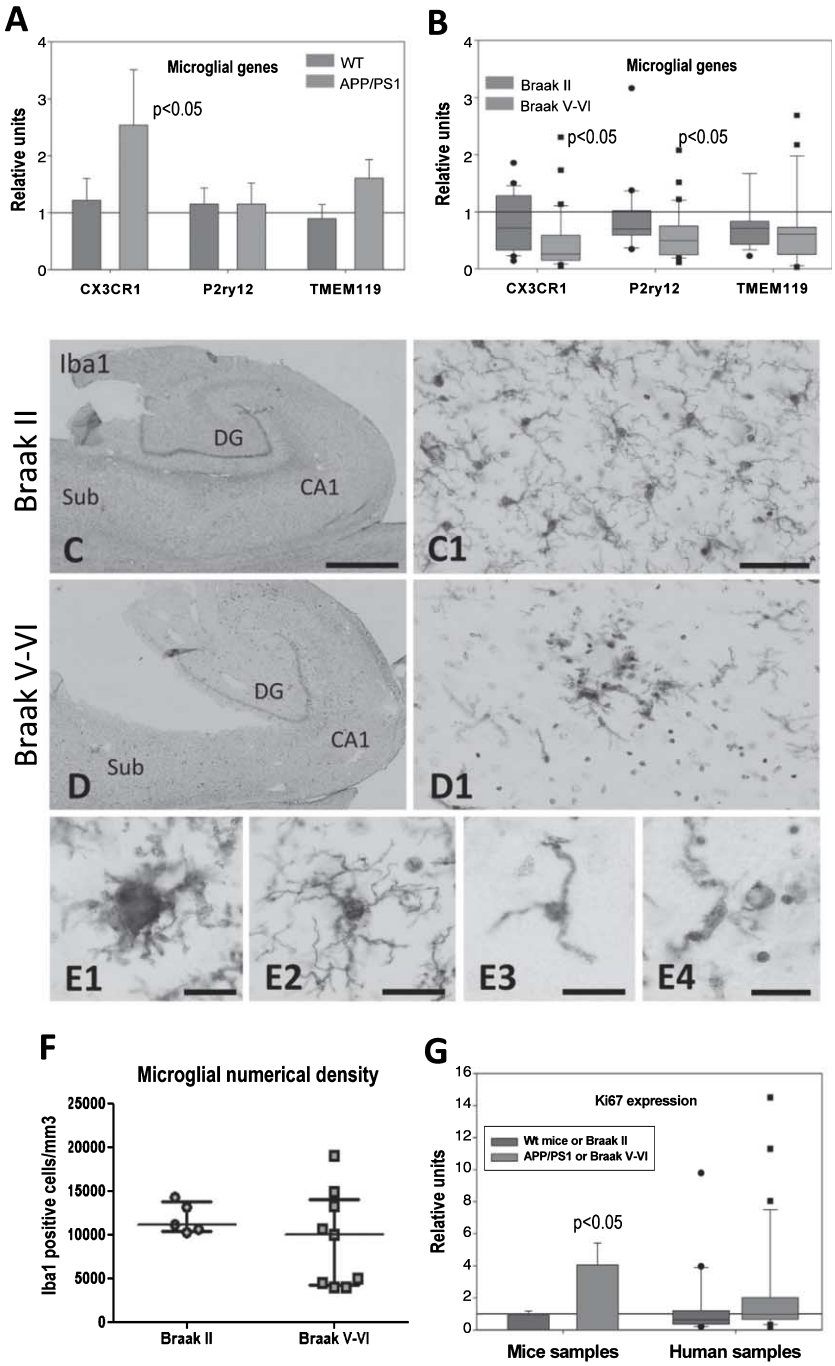

Fig. 3. Microglial degeneration in the human AD hippocampus. Microglial genes were analyzed by qPCR in the hippocampus of WT and AβPP/PS1 mice ($n = 10$ per genotype) (A) and human postmortem samples with Braak II ($n = 21$) or V-VI pathology ($n = 28$) (B). Representative images of Iba-1 immunostaining in the hippocampus of Braak II (C, and a detail in C1) and Braak V–VI cases (D, and a detail in D1); sections were immunostained with anti-Iba-1 and counterstained with cresyl violet. E) Iba1-positive microglial cells showing an activated (E1), non-activated (E2), and degenerated (E3 and E4) morphology; abnormal morphological features of Braak V-VI microglial cells included deramification (E3) and beading with spheroidal swellings (E4), of the processes. F) Numerical density (cells/mm^3) of Iba-1-positive microglia at the hilar region from Braak II ($n = 5$) and Braak V–VI ($n = 9$) cases was determined by stereology. G) Expression of the proliferative marker Ki67 by qPCR in the hippocampus of AβPP/PS1 mice and human AD brains compared to age-matched WT mice or Braak II samples, respectively. Significance was analyzed by two-tailed *t*-test (for mice samples) or Mann Whitney test (for human samples). CA1, hippocampal field; DG, dentate gyrus; Sub, subiculum. Scale bars: C and D, 2 mm; C1 and D1, 50 μm; E1-E4, 20 μm.

of toxic proteins (such as phospho-tau) together with a low proliferative capacity of the microglial cells in AD patients should produce the decrease of these cells in, predominantly, the dentate gyrus [37].

In sum, on the contrary to the classic view that microglia are highly stimulated by Aβ (as seen in AβPP-based models) and, in consequence, it could be implicated in the neurodegenerative process, our data demonstrate that the microglial response in AD brains is really mild. Moreover, and on contrary to amyloidogenic mouse models, in Braak V-VI subjects there is a prominent microglial degenerative process that, indeed, could compromise their normal role of surveying the brain environment and respond to the damage. This microglial degeneration, particularly relevant in the dentate gyrus of the hippocampal formation, might be mediated by the accumulation of toxic phospho-tau species. Hence understanding the multi-faceted nature of neuroinflammation in AD will lead to potential therapies capable of correcting dysregulated inflammatory responses and restoring cognitive function in AD patients.

CONCLUSION

Our work highlights relevant differences in the hippocampal inflammatory response elicited by AD mice and patients regarding microglial gene expression, morphology and survival. These differences need to be considered when delineating animal models that better integrate the complexity of AD pathology and, therefore, guarantee the translation of the research to the human brain.

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Role of Neuroinflammation in the Trajectory of Alzheimer's Disease and *in vivo* Quantification Using PET

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Abstract. Recent evidence suggests that neuroinflammation and immunity play a significant role in Alzheimer's disease and other neurodegenerative diseases. It has also been observed that, independent of the presence of aggregated proteins, neuroinflammation could be present in different neurodegenerative diseases. It has also been suggested that neuroinflammation could occur well ahead of amyloid deposition in AD. Recent genetic studies and other preclinical studies specifically point to a role of neuroinflammation and, in this review, we evaluate the evidence of neuroinflammation in the Alzheimer's disease trajectory and the different imaging modalities by which we could monitor neuroinflammation *in vivo* in humans.

Keywords: Alzheimer's disease, astrocytes, microglia, neurodegeneration, neuroinflammation

INTRODUCTION

Neuroinflammation is an innate response in the central nervous system (CNS) against harmful changes in brain milieu such as formation of abnormal protein aggregates, invasion of pathogens, traumatic and vascular lesions, and autoimmune responses to brain material such as myelin. It has been proposed that intrinsic neuroinflammation in the form of glial activation is a component of neurodegenerative diseases such as Alzheimer's disease (AD) and other dementias, Parkinsonian disorders, and Huntington's disease. While there are several mediators of inflammation which lead to neuronal damage, the pro-inflammatory cytokines and interleukin-1 β, IL-1, IL-8, and IL-33 play a significant role.

While neuroinflammation is a response that involves all cells present within affected region of the CNS, including neurons, microglia, and other inflammatory cells, there are several factors which influence how neuroinflammation affects the neurodegenerative process. These include environmental factors, previous immune sensitization, genetic factors, epigenetic factors, and several intrinsic and extrinsic factors. Preclinical models have shown that lipopolysaccharide (LPS) can induce toll-like receptor protein (TLR) signaling, which activates several signal transduction pathways including protein kinase B, mitogen protein activated kinase, and mammalian target of rapamycin in turn activating NF-κb. NF-κb mediates production of cytokines, chemokines, inducible nitric oxide

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synthase and cox2 promoting neurodegenerative processes.

There are several players involved in intrinsic neuroinflammatory process: these include microglia, astrocytes, oligodendrocytes, and inflammatory mediators such as cytokines, chemokines, and LPS.

MICROGLIA

At rest, microglia regulate the homeostasis of the brain but, if activated, become the resident macrophages of the CNS involved in the immune defense mechanism. They account for 10–15% of the non-neuronal brain cells. There is significant controversy regarding the precise nature of microglial progenitors. It has been suggested that microglia could arise from intrinsic brain embryonic progenitor cells. It has also been proposed that they could originate from meningeal macrophages penetrating the brain during embryonic development. There is still uncertainty about what proportion of the microglia are derived from blood monocytes and it is possible that monocytes may be recruited to the neonatal and adult brain when there is an injury and then differentiate into microglial cells. While there is controversy regarding the origin of microglial cells, the consensus is that microglial differentiation occurred primarily in the CNS [1]. While it has been shown that microglial progenitors invade the brain in the early stages, it is now established that microglia arise from the yolk sac [2]. Microglia serve as a part of the innate immune system which is constantly scanning and surveying signals for any danger to the brain cells. As a primary response to injury, microglia become activated in order to protect the CNS from tissue damage and facilitate tissue repair and clearance. Microglia also contribute to the control of neuronal proliferation and differentiation and influence synaptic connections. It has been shown that there is an interaction between the microglia and synaptic connections in the healthy brain. Microglia help regulate the wiring of the brain circuits allowing adaptive recovery processes to occur and control the growth of dopaminergic axons and neocortical neurons [3, 4].

While the origin of microglia has been debated, it seems clear that microglia are of monocyte lineage and present in the brain from birth. They are the resident macrophages of the CNS which constantly survey the brain to maintain normal homeostasis. During normal homeostasis, they maintain the plasticity of the neuronal circuit and contribute to the protection and remodeling of the synapses. It has been suggested this protective effect of microglia is mediated by the release of trophic factors such as brain-derived neurotrophic factors which are implicated in memory formation. Resting microglia exhibit a highly ramified morphology, which can exceed 50 μm in length, suitable for monitoring the environment. In response to an activating signal, they begin to withdraw the ramified branches (the withdrawal stage). When these processes are withdrawn, new protrusions may appear (the transitional stage) and then move on to a motile stage where the newly generated protrusions can grow and shrink at a rate exceeding 4 μm per minute. These motile cells begin to contact the neighboring cells and, during the motile stage, microglia can move through the tissue at the rate of 110 μm per hour and engulf other cells [5].

It has also been established that, while microglia engulf dead cells and cellular debris, they can also transiently ensheath a cell sized object and then move on without ingesting the object. These transient ensheathing events indicate their dynamic nature and possible role in tissue surveillance. It is proposed that this transient ensheathing (frisking), where the frisked object may very well be neurons or other cells which maintain the normal milieu of the brain plays a protective role as does the microglial responsibility for clearance of Aβ and other toxic proteins from the brain.

Amyloid-β (Aβ) clearance is an important process of microglial function. In AD, microglia can bind to soluble Aβ oligomers and Aβ fibrils via cell-surface receptors. The cell surface receptors include CD36, CD14, α6 β1 integrin, CD47 and TLR (TLR2, TLR4, TLR6, and TLR9). It has been shown that CD36, TLR4, and TLR6 trigger a pro-inflammatory response while binding Aβ. Further experiments have demonstrated that deletion of CD36, TLR4, and TLR6 reduces Aβ induced cytokine production and amyloid accumulation.

Microglia engulf Aβ fibrils by phagocytosis whilst soluble Aβ is degraded by various extracellular proteases [6]. Microglia contribute to CNS homeostasis and neuroprotection during development by synaptic pruning and phagocytosis of redundant neurons. They are involved in cortical laminar formation and axon bundle fasciculation. It has been shown that peripherally delivered lipopolysaccharide (LPS) can activate TLR receptors on the luminal surface of brain endothelial cells, which then secrete cytokines and activate microglia. It has been demonstrated that activated microglia can strip axosomatic inhibitory

synapses from neuronal soma which induces neuroprotection by upregulation of BCL1, FGF2, or MCL1, which are anti-apoptotic molecules. These microglia can assume an M2-AP phenotype able to secrete ceruloplasmin, CD163, SAA3, YM-1, and MSR1 during the initial phase of neuronal injury [7].

It is generally accepted that phenotypes of microglia fall into two main classes: 1) A pro-inflammatory or M1 phenotype which is activated the classical complement pathway and changes in brain milieu; and 2) An anti-inflammatory or M2 phenotype which is activated by the alternative complement pathway. The M1 phenotype responds to LPS in combination with interferon gamma (IFN-γ), leading to a massive inflammatory response producing cytokines including interleukin-1β, IL-12, TNF-α, and inducible nitric oxide. The M2 phenotype has three sub-phenotypes, M2a, which usually responds to IL-4 and IL-13, while M2b is stimulated by TLR or IL-1β activation. M2c represents the deactivated macrophages and contributes to the suppression of pro-inflammatory cytokines [8, 9]. It has been suggested that these phenotypes can interconvert depending on the stimuli and so models based purely on inducing an M1 or M2 phenotype is over simplistic. Despite this, a simplified model where pro-inflammatory phenotypes are regarded to be predominantly detrimental while anti-inflammatory phenotypes are regarded as predominantly involved in the repair process of the neurons has proved useful.

While there is evidence to suggest microglial activation can be deleterious, the beneficial effect is highlighted in circumstances where repair is happening, as in after stroke, during myelin repair, removal of toxic aggregated proteins and cell debris from the CNS, as well as secretion of neurotrophic factors to prevent neuronal injury [10–12].

While it is agreed that the M1/M2 microglial classification is an over simplified model, these two phenotypes have been studied extensively in cell culture and it has been demonstrated that the relative populations have differential influences over pathological outcome in CNS human diseases.

ASTROCYTES

Astrocytes are glial cells characterized by star-shaped cell bodies with a number of processes. There are two types of astrocytes: 1) Protoplasmic astrocytes, which are found in the grey matter and their processes end in sheet like appendages; and 2) fibrous astrocytes, which are found in the white matter and have long fine processes. While the function of astrocytes is still debated, it is generally thought that they provide nutrition for neurons and insulate nerves and synaptic connections from each other. They help regulate the potassium concentration in the space between the neurons [13]. More importantly, they perform the housekeeping chores that promote efficient signaling between neurons and they maintain surrounding neurons by releasing growth factors.

Astrocytes enfold all the blood vessels of the brain and ensheath synapses. As their physical association with synapses is closer than 1 μm, astrocytes can regulate local extracellular concentration of ions, neurotransmitters, and other molecules. The pathological response of astrocytes is reactive astrogliosis forming scars whereas remodeling of astrocytes is generally aimed at neuroprotection and recovery of injured neuronal tissue [14, 15]. Reactive astrocytes are characterized by increased expression of glial fibrillary acidic protein (GFAP). However, many healthy astrocytes do not express detectable levels of GFAP and the expression of GFAP can depend on the anatomical location of the astrocytes as well as the species in which GFAP expression is being examined. Aging is the leading risk factor for the common dementias, and astrocytes in the aging brain show features of senescence and expression of a senescence associated secretory phenotype.

Initially it was thought that astrocytes appeared activated in AD brain as a secondary or non-specific response to the disease process [16]. However, it is now understood that astrocytes are central to the pathogenic mechanism in neurodegeneration. This could be due to their production of cytokines and chemokines or loss of physiological functions such as neuronal support and spatial buffering. It has been suggested that disruption of normal glioneuronal interaction can lead to synaptic dysfunction and contribute to cognitive impairment [15, 17]. Wyss-Corey et al. first demonstrated *in vitro* that astrocytes are able to take up and degrade Aβ using cultured mouse astrocytes. Histopathological studies of AD brain have shown the presence of astrocytes which contain Aβ suggesting they are involved in the clearance of this peptide [18–21]. Engulfment of Aβ by astrocytes, however, can lead to their death and give rise to secondary plaques [21].

The mechanisms governing the receptor-mediated uptake of Aβ and its consequences are not fully

understood. For instance, does uptake of Aβ induce a change in astrocyte phenotype altering their usual neurosupportive function? Low density lipoprotein receptor-related protein 1 is involved in the uptake and clearance of Aβ and is also a receptor for the uptake of ApoE4 and complexes of ApoE-Aβ highlighting the importance of this receptor in the astrocytic clearance of Aβ [22–24].

Neuroinflammation is a prominent and early feature of AD which plays a key role in modulating the progression of disease via inflammatory mediators and neurotoxic compounds. It has also been suggested that an astrocyte mediated inflammatory response can contribute to the neurodegenerative process through expression of pro-inflammatory cytokines and chemokines, activation of complement cascade as well as reactive oxygen and nitrogen species [25–27]. Studies also show that astrocytes can suppress innate immunity through αB-crystallin suggesting that they have a more deleterious influence on neuroinflammation. In animal models of AD, it has also been shown that the astrocyte contribution to neuroinflammation is significant and an important therapeutic target [28, 29].

Apart from microglia and astrocytes, other cells such as blood derived monocytes may also play a significant role in AD. However, the precise role of these cells in human studies is unclear although there are animal studies demonstrating infiltration of these peripheral mononuclear cells associated with amyloid deposition. Ablation of CD11b-positive cells in APP/PS1 models of AD have suggested that peripheral monocytes do play an important role in clearing amyloid plaques [11, 30].

P2X7 RECEPTOR

The purinergic P2X7 receptor (P2X7R) plays an important role in the CNS binding ATP. The P2XR is expressed by activated microglia and, following brain injury, ATP can be released in large quantities leading to stimulation of low affinity P2X7Rs resulting in glial necrosis/apoptosis or proliferation, demonstrating two opposing effects of neuroinflammation [31, 32].

P2X7Rs or ATP-gated non-selective cation channels are made up of 595 amino acid subunits. The common structural motifs of P2X7R are the two transmembrane domains and a large glycosylated cysteine-rich extracellular loop as short intracellular and terminal domain and intracellular C-terminal domain [33–35]. Activation of P2X7R results in the opening of the channel pore, allowing the passage of small cations (Na^+, Ca^+, and K^+). Additionally, P2X7 is characterized by opening of a non-selective pore in response to repeated or prolonged activation, allowing permeation of larger molecular weight organic cations up to 600–800 Da. Patency of the large pore eventually results in membrane blebbing and cell death [36–38].

Cytokines are the major mediators of neuroinflammation, including pro-inflammatory and anti-inflammatory processes, chemo-attraction and Aβ deposition in response to microglial activation. It has also been suggested that, as Aβ concentration increases in aging transgenic mouse models, it is associated with increased levels of the cytokines TNF-α, IL-6, interleukin 1-α, and GM-CSF, suggesting pathological accumulation of Aβ could drive a neuroinflammatory response [39–41].

Chemokines regulate microglial migration to areas of neuroinflammation and enhance local inflammation in AD. There is upregulation of CCL2, CCR3, and CCR5 expression by microglia, whereas CCL4 is expressed by reactive astrocytes. It has been shown that Aβ deposition leads to generation of interleukin-8, CCL2, and CCL3. It has also been suggested that CX3CR1/CX3CL1 is involved in neuronal survival, plaque load, and cognition [42–44].

The complement system is a major constituent of the immune system in the defense against pathogens. Activation of the proteolytic complement cascade results in opsonization. Major sources of complement system proteins include microglia and, to a lesser extent, astrocytes [45, 46]. It has also been shown that Aβ can activate the complement pathway. The protein clusterin is involved in the processing and clearance of immune complexes and is also a regulator of C3 convertase activity. Raised clusterin levels are associated with an increased risk of AD.

PET IMAGING OF NEUROINFLAMMATION

Studies have shown microglial activation to be a component of many CNS disorders including multiple sclerosis, focal epilepsy, stroke, and brain tumors. It is clear that all neurodegenerative diseases are associated with significant levels of neuroinflammation but that this inflammatory process is different from the autoimmune diseases of brain. While in relapsing remitting multiple sclerosis, it has been

shown that the inflammatory process is accompanied by T-cell activation with specificity for CNS antigen infiltrates, the inflammatory reaction in AD is associated with activation of microglia in close proximity to Aβ plaques [47, 48]. During the activation process, microglia express the translocator protein (TSPO)—previously known as the peripheral benzodiazepine receptor (PBR)—on the outer surface of their mitochondria. This protein binds isoquinolines such as PK11195, and diazepam, and is present in peripheral tissues such as kidney, liver, and lungs. It was later demonstrated that TSPO/PBR is different from the central benzodiazepine receptor which is a component of the $GABA_A$ complex found in the CNS [49]. TSPO forms a multimeric complex with a 32 kDa voltage dependent anion channel called mitochondrial porin and 30 kDa adenine nucleotide carrier in the outer mitochondrial membrane [49, 50]. Recent studies have shown that TSPO transports cholesterol, anions, and other substrates across the mitochondria and helps maintain the membrane potential [51, 52].

The enzyme monoamine oxidase B (MAO-B) is expressed by astrocytes and hydrolyses trace amines, phenylethyl amine, and dopamine. It binds deprenyl and D2-deprenyl. MAO-B expression increases with age and is thought to contribute to age-related neurodegeneration. Astrocytes upregulate expression of MAO-B under physiological and pathological conditions and so levels of brain MAO-B reflect astrocytosis.

Arachidonic acid is a polyunsaturated omega-6 fatty acid present in the phospholipid bilayer membranes in the brain. It serves as a second messenger and is involved in the upregulation of several signaling enzymes. It is now considered that arachidonic acid plays an important role in the inflammatory process. It has been suggested that binding of cytokines derived from microglia to calcium channel receptors on astrocytes activates phospholipase enzyme that releases arachidonic acid from membrane lipoproteins. Owing to these properties, arachidonic acid has been suggested to be a useful marker of neuroinflammation. Additionally, cyclooxygenase catalyzes the breakdown of arachidonic acid into prostaglandins. There are two isoforms of cyclooxygenase, Cox-1 and Cox-2, where Cox-1 is predominantly found in microglia whereas Cox-2 is expressed postsynaptically in neurons in the cortex, amygdala, and hippocampus [53]. Cox is also involved in the inflammatory cascade and is thus considered as a biomarker for neuroinflammation.

IMAGING TRANSLOCATOR PROTEIN

There are several TSPO radioligands which have been used to detect microglial activation *in vivo* in humans.

The TSPO radiotracer that has been most widely used is [^{11}C]-R-PK11195, an isoquinoline [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide]. It is a selective antagonist for TSPO and, during the development of this radiotracer, it was shown that the R-enantiomer has two-fold higher affinity for TSPO compared to the S-enantiomer. Initial studies with PK11195 were conducted more than two decades ago, following which many papers have been published demonstrating neuroinflammation can be detected in a variety of neurodegenerative diseases and in neuroinflammatory conditions [54–57].

Cagnin et al. reported the first AD study with [^{11}C]-PK11195 PET and reported up to 40% increases in temporal lobe binding using a region of interest approach [58]. Increased microglial activation with aging was also seen. Subsequent studies generally confirmed the increased [^{11}C]-PK11195 uptake in AD brain, though some studies failed to detect this [59–61].

Studies have also evaluated the relationship between amyloid load and neuroinflammation. It has been reported that neuroinflammation correlates with amyloid load in AD and mild cognitive impairment (MCI) cases with raised amyloid deposition. While clusters of significant correlation between amyloid deposition and neuroinflammation have been demonstrated, these studies have also shown regional discrepancy between these two pathological processes, suggesting that neuroinflammation could also be triggered by other pathologies such as tau tangles and alpha synuclein aggregation. It has also been pointed out that, while we are able to image amyloid deposition using imaging ligands for beta sheeted protein, we are still unable to detect oligomeric Aβ which is most likely the toxic species contributing to the microglial activation [62, 63]. The trajectories of amyloid aggregation and microglial activation are likely to be different as the first precedes the second and rises to a stable level while inflammation rises and then may fall. Whether correlations are seen between amyloid and PK11195 uptake in brain regions may depend on the time point of the disease. While positive correlations have been detected between PK11195 and PIB uptake, one group could not demonstrate any such

correlation, while another group found a negative correlation between amyloid and neuroinflammation [60, 64]. Longitudinal studies on preclinical and prodromal AD cases are really needed to sort this inter-relationship out. As other pathologies could also contribute to neuroinflammation, the advent of tau imaging agents is now allowing groups to evaluate the inter-relationships between amyloid, tau, and neuroinflammation.

While there have been discrepancies between the results across centers, it is now generally accepted that there is increased cortical microglial activation in AD most closely tracking an amyloid pattern. Several recent studies have also shown that there is increased microglial activation in amyloid positive MCI subjects and that this microglial activation can be seen before the conversion to dementia. Measurement of microglial activation using PK11195 PET shows increased regional tracer binding in the entorhinal, temporoparietal, and cingulate cortex in AD and MCI subjects. It has also been shown that microglial activation is increased in Parkinson's disease, Parkinson's disease with dementia, Lewy body dementia, schizophrenia, traumatic brain injury, multiple sclerosis, stroke, and several neuroinflammatory diseases. Despite the demonstration of microglial activation in these conditions using PK11195 PET tracer, there has been considerable controversy over its utility because of its relatively low signal-to-noise ratio. This has led to the development of several novel second-generation TSPO PET radiotracers with higher affinity and lower background signals. These include [^{11}C]-PBR28, [^{11}C]-DAA1106, [^{11}C]-DPA713, [^{18}F]-FEDAA1106, [^{18}F]-PBR06, [^{18}F]-FEPPA, [^{18}F]-DPA-714, and [^{18}F]-GE180. [54, 65–70]

These second-generation TSPO PET tracers were developed to overcome the shortcomings of [^{11}C]-PK11195. However, one of the main limitations of the second-generation TSPO tracers is that their binding is influenced by the TSPO polymorphism expressed by subjects leading to differential binding across the general population due to variations in TSPO binding affinity. A polymorphism on the TSPO gene consisting of one amino acid substitution (Ala147Thr) results in the population having a high affinity binding (HAB) phenotype, mixed affinity binding (MAB) phenotype, or low affinity binding (LAB) phenotype for TSPO ligands other than PK11195. The Ala/Ala TSPO genotype (wild-type) results in HAB, while the Ala/Thr results in MAB, and Thr/Thr results in LAB. It has been shown that roughly 50% of the general population are high affinity binders while around 40% are mixed affinity binders and 10% of the population are low affinity binders. Hence, for a homogeneous population, one should select high and/or mixed affinity but not low affinity binders for study with 2nd generation TSPO tracers [71]. While concerns have been raised regarding the utility of the studies conducted in subgroups of the population, it has been demonstrated that, in AD and MCI subjects, studies performed in apoE4 or apoE3 genetic subgroups could be generalized to the entire AD/MCI population, at least in observational studies. However, one could speculate that this could hold true even in intervention studies, as long as the treatments were not influencing cholesterol metabolism.

Studies using [^{11}C]-PBR28 have shown a very high specific signal for microglial activation with an increased 80-fold affinity in animal models. Studies in AD subjects demonstrated that there is increased microglial activation specifically in the inferior parietal lobule, precuneus, occipital cortex, hippocampus, and entorhinal cortex [68, 72]. However, surprisingly these workers were unable to detect inflammation in amyloid positive MCI cases.

Despite the significant interest in the second generation tracers, results using [^{11}C]PBR28 have been inconsistent. While some groups have been able to demonstrate a significant difference between the AD and healthy control subjects, other groups were unable to show consistent differences. The average percentage increase in AD subjects compared to the control subjects was similar to that seen with [^{11}C](R)PK11195 PET (around 30%). While no head-to-head study has compared [^{11}C]PBR28 and [^{11}C](R)PK11195 PET in AD, there is no convincing evidence to suggest that one tracer is more sensitive than the other. As there is no typical reference devoid of microglial activation in the brain in neurodegenerative diseases, TSPO ligands are also affected by the quantification issues. While supervised cluster analysis has been used to define a reference tissue cluster representing normal grey matter uptake kinetics for [^{11}C](R)PK11195, such an approach has not been feasible for [^{11}C]PBR28. Hence, a cerebellar reference has been used to reflect non-specific uptake approach for [^{11}C]PBR28 which is likely to overestimate this component. There is also considerable variability in the plasma protein binding of TSPO ligands across subjects and disease states [73-75]. This makes using an arterial plasma input reference function difficult due to the variability in time activity curves.

Initial studies with [^{11}C]-DPA-713 demonstrated that it provided better sensitivity than [^{11}C]-PK11195 and showed more TSPO density in widespread regions of ageing subjects and also AD subjects [76, 77].

While [^{11}C]-DPA-713 was being evaluated, a newer, higher affinity, higher specific to non-specific binding tracer with a longer half-life, [^{18}F]DPA-714 was developed and evaluated [66, 78]. [^{18}F]DPA-714 has demonstrated significant increases in the frontal, temporal, and parietal cortex of AD cases, again suggesting that microglial activation could be detected with both first and second generation TSPO tracers. Interestingly, highest tracer binding was seen in prodromal AD suggesting that inflammation may reduce as MCI progresses to AD.

Other second-generation tracers include [^{18}F]FEPPA, where PET has shown that there is significant uptake in the grey matter of the hippocampus, prefrontal cortex, temporal, parietal, occipital cortex, posterior limb of internal capsule, and cingulum of AD cases [79, 80]. [^{18}F]FEMPA PET detected significant uptake in the medial and lateral temporal cortex, posterior cingulate, caudate, putamen, and thalamus in AD. [^{11}C]DAA-1106, [^{18}F]FEDAA-1106, and [^{11}C]vinpocetine PET have also demonstrated significant microglial activation in AD and other neurodegenerative diseases [60, 65, 81].

In the early stages of AD (amyloid-positive MCI subjects), [^{11}C]DAA-1106 and [^{18}F]DPA-714 PET have demonstrated high increases in binding in the frontal, temporal and parietal cortex. This was consistent with previous observations using [^{11}C](R)PK11195 PET [82]. While initial studies with [^{11}C]-PBR28 failed to demonstrate increased microglial activation in MCI subjects, recent studies have shown that increased microglial activation in MCI subjects can be seen on a single subject analysis.

Microglial activation has been reported in AD variants such as posterior cortical atrophy, where PBR28 has demonstrated significantly increased binding in occipital, posterior parietal, and temporal regions. While there have been some reports concerning correlation with age, later reports using [^{18}F]DPA-714 in a larger cohort have not shown an age effect. There has been significant negative correlation between TSPO binding and cognitive performance using [^{11}C]-PBR28, [^{11}C](R)PK11195 and [^{18}F]FEPPA. Interestingly, a recent study in a large number of AD and MCI subjects demonstrated that MMSE was positively correlated with microglial activation [70]. Studies have already demonstrated that, in AD subjects, there is a negative correlation between microglial activation and atrophy while, using [^{18}F]DPA-714, microglial activation was positively correlated with the grey matter volume in MCI and AD patients. Studies have already demonstrated correlations between amyloid load using [^{11}C]PIB and [^{11}C](R)PK11195, [^{11}C]PIB and [^{11}C]PBR28 and [^{18}F]DPA-714 in different cortices, precuneus, hippocampus, and parahippocampal gyrus.

LONGITUDINAL EVALUATION OF MICROGLIAL ACTIVATION

There are only a handful of studies which have evaluated the longitudinal relationship of microglial activation and disease progression. Fan et al. demonstrated that there is increased microglial activation as the disease progresses in established AD, while in MCI subjects there was a longitudinal reduction. Please see Fig. 1. The authors argued that the microglial activation detected by TSPO tracers in the early and late stages of the disease could be phenotypically different, and in the early stage of the disease it may be detecting the anti-inflammatory phenotype while during the later stages of the disease it may be detecting the pro-inflammatory phenotype. It has also been suggested that, while the anti-inflammatory phenotype becomes ineffective in clearing amyloid and toxic debris, there is progressive amyloid deposition and neuronal damage. In contrast, as the disease progresses there is persistent activation of the pro-inflammatory phenotype which is also detected by the microglial tracer as a persistent elevation of microglial activation. This later phase of microglial activation is also detected by the TSPO tracer and continues to rise as the disease progresses and correlates with the cognitive impairment [83].

Kreisl et al. demonstrated that in AD subjects there is increased binding of [^{11}C]-PBR28 in the inferior parietal lobule, precuneus occipital cortex, hippocampus, entorhinal cortex, middle and inferior temporal cortex. Longitudinally there was an annual increase of 3.9 to 6.3% in patients with AD. It is also proposed that the annual rate of increased TSPO binding in the tempoparietal region was about five-fold higher in patients with clinical progression compared to those who did not progress [84].

Hamelin et al. evaluated 64 patients with AD and 32 controls. They demonstrated that higher microglial

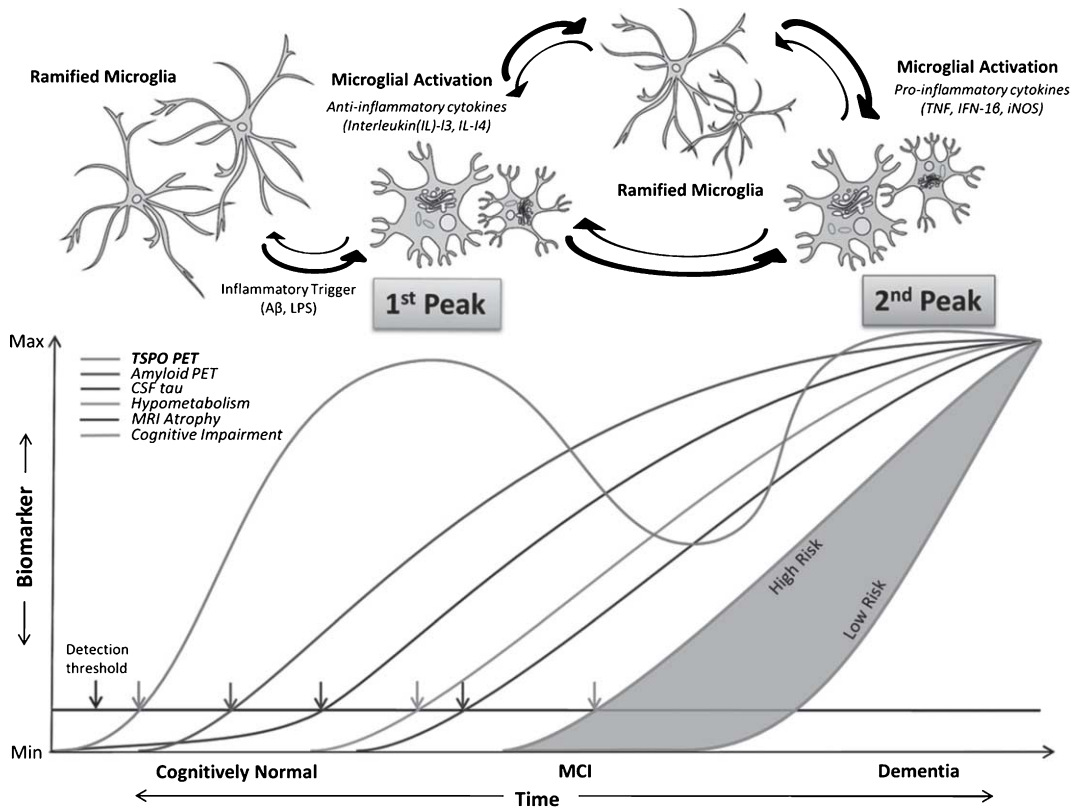

Fig. 1. Hypothetical model of dual peak of microglial activation in the Alzheimer's disease (AD) trajectory. The upper panel demonstrates the hypothetical model of morphological changes in microglia in AD trajectory, where ramified microglia transform to anti-inflammatory (protective) microglial phenotype and pro-inflammatory (toxic) microglial phenotypes. The lower panel shows the microglial activation in relation to other biomarkers detectable using positron emission tomography where two peaks of microglial activation are present in AD trajectory (Reprinted from *Brain* [83]).

activation was present in slow decliners compared with fast decliners. They also demonstrated that microglial activation is present in prodromal and possibly at the preclinical stage of AD and was found to play a protective role in in the clinical progression of the disease. This study further substantiates the concept that microglial activation could be protective in early stages of the disease [70].

IMAGING ASTROCYTE ACTIVATION

L-deprenyl is an irreversible monoamine oxidase-B (MAO-B) inhibitor, which exists on the outer mitochondrial membrane of astrocytes [85, 86]. The radiotracer [^{11}C]deuterium-L-deprenyl ([^{11}C]DED) has high affinity and specificity for MAO-B increases in most brain regions in healthy older adults. Activity of MAO-B increases in AD patients' brains where the enzyme is over expressed by reactive astrocytes. Autoradiographic studies have demonstrated that [^{11}C]DED can be used as an *in vivo* PET ligand for assessing MAO-B in AD brains. In a study of eight MCI subjects, seven AD subjects, and 14 healthy controls it has been shown that there is increased astrocyte activation in the left temporal, left insular cortex, bilateral anterior cingulate, right parahippocampal cortex, right hippocampus, right caudate, and left putamen [87]. It was also shown that increased [^{11}C]DED binding to MAO-B was more evident in the amyloid-positive MCI subjects compared to the amyloid-negative subjects and AD subjects.

Novel astrocyte markers are being tested which includes markers of imidazoline binding. Preliminary data using the tracer [^{11}C]BU99008 have demonstrated significant uptake in different cortical regions in healthy control subjects. While the results of further studies are awaited, the results from the healthy control subjects are promising.

While it is recognized that neuroinflammation is a prominent and early feature of AD which plays a key role in modulating disease progression, the role of astrocyte activation is still being debated. Several studies indicate that astrocyte-mediated inflammatory processes also contribute to neurodegeneration in AD through increased astrocytic expression of pro-inflammatory cytokines and chemokines, activation of the complement cascade as well as reactive oxygen and nitrogen species. To understand the role of astrocytes, further studies are necessary using *in vivo* imaging agents which would allow us to track the progression of astrocyte activation longitudinally.

NOVEL TARGETS OF NEUROINFLAMMATION

Apart from targeting TSPO, further work is necessary to develop new targets to detect the migratory capacity of microglia or their ability to phagocytose toxic products. While such targets could be of very significant interest, new approaches such as cell type specific transcriptional profiling and identification of numerous cell specific changes may provide a challenge and is being still pursued as a novel strategy to identify microglial activation.

The cannabinoid type 2 receptor (CB2R) is part of the endogenous cannabinoid system which is an alternative membrane marker of microglial activation. PET tracers showing high affinity for CB2R have been developed, one of which is [^{11}C]NE40. However, this tracer showed lower uptake in AD patients compared to the control subjects. It was suggested this could be due to low level of CB2R expression and insufficient selectivity for CB2R. Several other high affinity agonists are also being evaluated as CB2R tracers, such as [^{11}C]MA2, [^{18}F]MA3, [^{18}F]RS126 [88, 89].

It has been shown that [^{11}C]KTP-Me is a pro-radiotracer for ketoprofen (KTP) and animal studies have suggested that [^{11}C]KTP is retained in inflammatory lesions due to the expression of Cox-1. While a first human study in healthy volunteers showed that [^{11}C]KTP-Me could be a potential PET tracer with good penetration in human brain, subsequent studies did not find a difference between controls and AD subjects [90]. Nicotinic acetylcholine receptors (nAChR) are upregulated in neuroinflammation. The ligand targeting $\alpha4\beta2$ nAChR has been demonstrated to have similar patterns of uptake as [^{11}C]-PK11195. However, despite the initial enthusiasm, several nicotinic acetylcholine receptor tracers have not been successful. New compounds such as [^{18}F]ASEM and [^{18}F]DBT-10 are now being evaluated [91].

Recent studies have shown that the P2X7 receptor is widely present in neuroinflammation. Studies have shown that deletion and pharmacological blockade of P2X7Rs alter responsiveness in animal models of neurological disorders. P2X7 receptors are expressed in the cell-surface membrane of hematopoietic cells such as macrophages and microglia. Novel PET tracers targeting P2X7 receptors include [^{11}C]GSK1482160, [^{11}C]A740003, and [^{11}C]JNJ-54173717.

Other targets of interest include phospholipase A2 (PLA2) activity. It has been shown that inflammatory cytokines released from microglia can bind to astrocyte receptors which are coupled to PLA2. When this enzyme is activated it hydrolyses arachidonic acid (AA) from the membrane. Hence, by measuring the brain uptake of [^{11}C]arachidonic acid, one could determine the metabolic loss of arachidonic acid in the brain. It was proposed that increased incorporation of [^{11}C]AA could represent upregulated AA metabolism due to neuroinflammation.

Another target is adenosine A2A receptors (A2AR). The binding of adenosine to A2AR tends to attenuate inflammation by endogenously limiting the inflammatory response and leads to upregulation of these receptors at the sites of inflammation. While these mechanisms have been proposed, to date no definite tracer which could replace the TSPO tracer has been developed.

CONCLUSION

It is now clear that neuroinflammation plays a significant role in AD and neurodegenerative diseases. Microglia and astrocytes play a significant role in neuroinflammation; however, activation of microglia and astrocytes can vary depending on the stage of the disease and the trajectories are still uncertain. While we are now able to image activation of microglia and astrocytes, further research is necessary to evaluate whether initially they have a protective and later a cidal influence on neurodegeneration as some series have suggested. There have been significant advances in imaging microglia, with further recent advances in imaging astrocytes. More evidence is emerging regarding the differential role of microglial and astrocyte activation in different stages of neurodegenerative disease, which will form the basis of

future research in neuroinflammation in the coming decades. As there are many other processes involved in neuroinflammation, future research will need to develop biomarkers to evaluate new markers such as chemokine receptor function to differentiate the pro-inflammatory and anti-inflammatory molecules involved in neuroinflammation. Current evidence suggests that not all the neuroinflammatory processes happening in the brain are detrimental, and further research is necessary to separate and understand them.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9929).

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Microglial Activation During Pathogenesis of Tauopathy in rTg4510 Mice: Implications for the Early Diagnosis of Tauopathy

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Abstract. Tauopathy is characterized by the fibrillar tau accumulation in neurons and glial cells. In order to advance our understanding of the causative mechanisms of tauopathy, neuroinflammation, which has been suggested to play important roles in disease progression, will require particular attention. Neuroinflammation is characterized predominantly by microglial activation. At present, it is still under debate whether microglial activation is a cause or a result of neurodegeneration. To search for a temporal relationship between neurodegeneration and neuroinflammation, our group demonstrated that *in vivo* imaging (e.g., tau-PET, TSPO-PET, and volumetric MRI) of tauopathy mice strongly supports the evidence of microglial activation along with both pathological tau accumulation and brain atrophy. Both *in vivo* imaging and histochemical analysis confirmed that microglial TSPO accumulation was the late event during the pathogenesis of tauopathy. On the other hand, it is known that purinergic receptor P2Y12 as a marker of homeostatic microglia cells was reduced at an early stage of disease progression. In this review, we will introduce a phenotypic change of microglia in a mouse model of tauopathy and propose novel approaches to the establishment of imaging biomarkers, thereby targeting the early diagnosis of tauopathy.

Keywords: Microglia, neuroinflammation, P2Y12 receptor, PET imaging, tauopathy, TSPO

INTRODUCTION

Neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein are a primary neuropathological feature of a number of neurodegenerative diseases, collectively termed tauopathies, including Alzheimer's disease (AD), progressive supranuclear palsy, corticobasal degeneration, Pick's disease, and familial frontotemporal lobar degeneration (FTLD) with underlying tau pathology (FTLD-tau) (reviewed in [1]). Although tau pathology was initially dismissed as a secondary event in AD that was not integral to the neurodegenerative process, the discovery of tau mutations associated with the tauopathy FTLD-tau demonstrated that tau dysfunction could directly result in neurodegeneration. However, we are still lacking understanding of the potential mechanisms underlying the cause of the diseases.

For the purpose of modeling human tauopathy by showing prominent intracellular deposition of tau protein and associated neuronal loss, several transgenic mouse lines expressing FTLD-linked mutant tau have been developed [2]. Among them, the rTg4510 mouse line is one of the popular tauopathy models presenting features of age-dependent neuropathology as well as neurodegeneration both induced by the overexpression of P301L mutated human tau in cerebral cortex and hippocampus [3].

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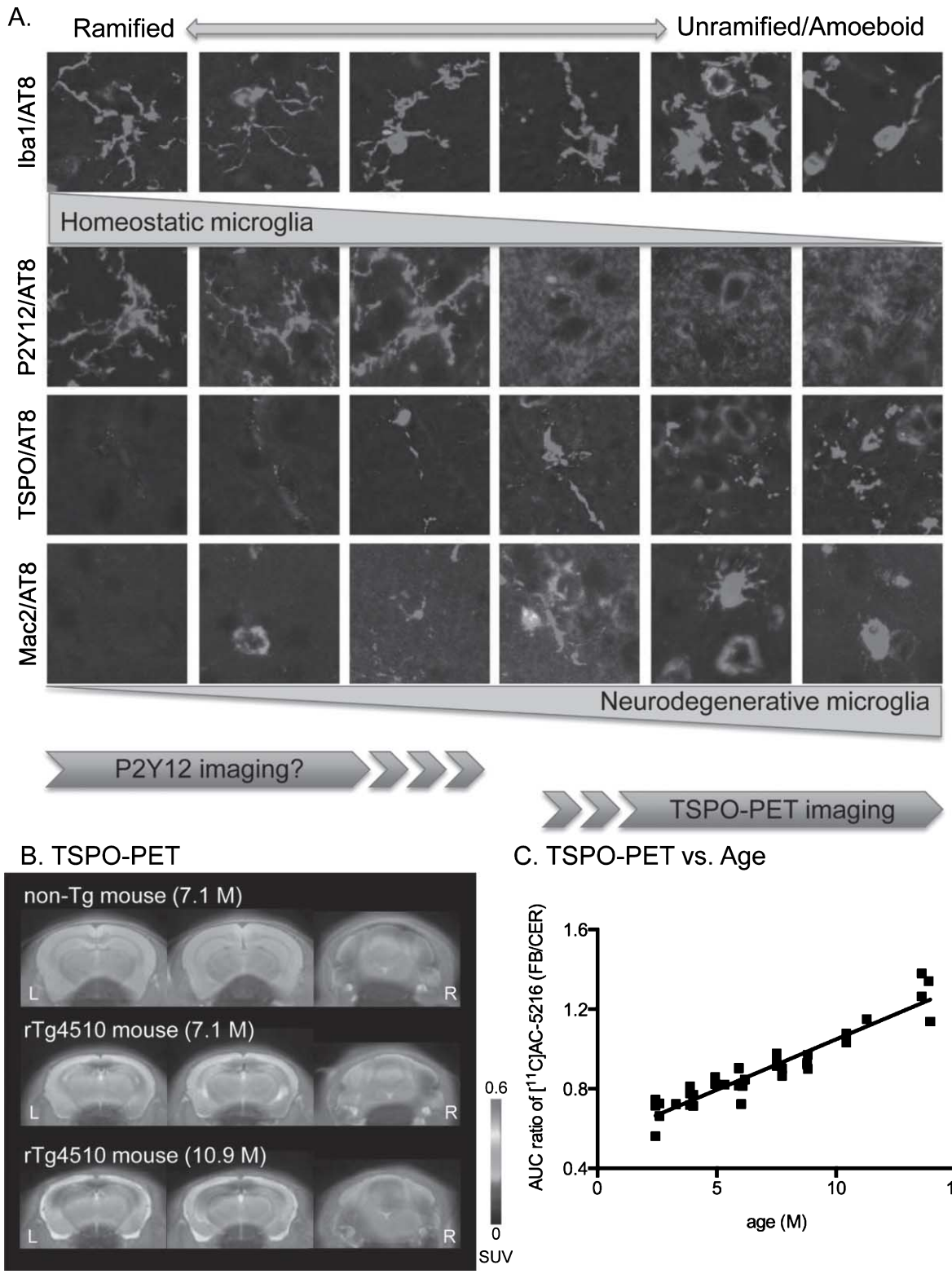

This mouse line is well studied not only for its application to cognitive impairment [3–5] but also for the advancement of tau positron emission tomography (PET) imaging [6, 7]. Especially, *in vivo* imaging studies have presented a new avenue for investigating causal mechanisms of neurodegenerative diseases via the monitoring of real-time brain functional changes.

Inflammation is considered a key factor in regulating both amyloid and tau pathologies, based on the fact that activated astrocytes and microglia are well associated with these pathological hallmarks (reviewed in [8]). Increasing numbers of studies have shown that microglia could become a chronic source of multiple neurotoxic factors (e.g., tumor necrosis factor-α, nitric oxide, interleukin-1β (IL-1β), and reactive oxygen species) [9]. Notably, it was suggested that microglial activation and resulting secretion of IL-1β triggered exacerbation of tau pathology [10–12]. A longitudinal cohort study (45–69 years old at the start of cognitive testing) showed that IL-6, a major proinflammatory cytokine [13], elevated in the sera of cognitively declined participants [14]. This also suggested that peripheral inflammation could contribute to neuronal damage. Furthermore, the immunosuppressive drug FK506 has been shown to attenuate the taupathology and increase the lifespan of the tau model PS19 mouse line, which expresses the P301S mutant human tau under control by the mouse prion promoter [15]. On the other hand, AD treatment trails using nonsteroidal anti-inflammatory drugs (NSAIDs) failed to demonstrate any clear clinical efficacy, suggesting that anti-inflammatory drugs may not be effective for the prevention of AD [16]. Nonetheless, the inflammatory mechanisms during the progression of neurodegenerative diseases are still largely obscure.

To determine whether microglial activation occurs prior to pathological tau accumulation, we have recently demonstrated longitudinal monitoring of both *in vivo* tau pathology and the mitochondrial 18-kDa translocator protein (TSPO), as a marker of microglial activation, in a tauopathy mouse model rTg4510 using small-animal PET imaging [7]. Our data showed age-dependent TSPO accumulation along with both pathological tau accumulation and brain atrophy. The rising phase of TSPO accumulation was relatively later than that of tau accumulation, suggesting that microglial activation might be downstream of the formation of pathological tau aggregates. However, the exact activated microglia state (e.g., change of cellular morphology, surface phenotype, secretory mediators, and proliferative responses (reviewed in [17]) has not been clearly understood. Here, based on our current studies of rTg4510 mice [7], we will introduce the temporal change of microglial phenotypes during the development of tauopathy. Furthermore, we will discuss the possible implications of *in vivo* imaging technologies for evaluating disease progression.

ACTIVATED MICROGLIA IN rTg4510 MOUSE BRAINS

The stages of microglial activation were defined based on morphological, molecular, and functional characteristics. To investigate microglial morphology, Iba1 (ionized calcium binding adaptor molecule 1) is the most reliable marker because it is expressed in microglia throughout various morphological states and is upregulated in activated microglia [18, 19]. As can be seen in the panels of microglial morphology from rTg4510 mouse brains, Iba1 immunostaining showed all types of microglial morphology (Fig. 1A). We recently demonstrated that Iba1 staining on rTg4510 sections showed age-dependent increases of unramified microglial cells in the cerebral cortex and hippocampus [7]. Double-labeling with Iba1 and TSPO antibodies further confirmed the increased activated microglia in 6-8-month-old rTg4510 mice [7]. This change was strongly associated with both pathological tau deposition and brain

Fig. 1. Morphological phenotypes and markers of microglia. A) Representative images of microglial morphologies were aligned from ramified shapes (left) to unramified/amoeboid shapes (right). Each morphology was labeled by the microglial markers Iba1 (rabbit polyclonal, Wako), P2Y12 (rabbit polyclonal), TSPO (rabbit monoclonal, Abcam), and Mac-2 (rat monoclonal, Cedariane) antibodies. Images were double-labeled with microglial marker (red) and AT8 (green). P2Y12 imaging and TSPO-PET imaging are anticipated to visualize distinct morphologies. B) Representative TSPO-PET imaging with [^{11}C]AC-5216 radiotracer in mouse brains. Images of [^{11}C]AC-5216 signals in brains of 7.1-month-old non-tg (top), 7.1-month-old rTg4510 (middle), and 10.9-month-old rTg4510 (bottom) mice were generated by averaged dynamic scan data. The images showed dorsal hippocampal level of coronal (left), ventral hippocampal level of coronal (middle) and cerebellum level of coronal (right) slices. SUV (standardized uptake value) was calculated by injected dose per tissue volume x body weight. C) Scatterplot of AUC (area under the curve of the time-activity curve) ratio (forebrain to cerebellum) for [^{11}C]AC-5216 signals against age (months) of rTg4510 mice ($n = 40$).

atrophy [7]. From the aspect of morphological phenotypes, microglia can transform into unramified and amoeboid shapes under pathological condition. Since Mac-2, a member of the galectin family of β-galactoside binding lectins, serves as a marker of a subtype of activated microglia [20], functional phenotypes of activated microglia can be examined by the immunoreactivity of Mac-2 antibody (Fig. 1A). In the aged rTg4510 mouse cerebral cortex, a number of roundish/oval microglia were labeled by Mac-2 antibody (Maeda et al. manuscript in preparation). In addition to the immunohistochemical observations, TSPO-PET imaging in live rTg4510 mice demonstrated age-dependent TSPO accumulation in the cerebral cortex of rTg4510 mice (Fig. 1B, C; PET imaging data and experimental procedures were also reported in [7]). Thus, the linkage between tau pathology and microglial activation was clearly confirmed by our multifaceted studies.

MICROGLIA STATUS IN EARLY STAGE OF TAUOPATHY

Microglial morphologies from ramified to unramified and amoeboid shapes were generally identified by Iba1 immunoreactivity (Fig. 1A). However, Iba1 immunolabeling may not be especially valuable for discriminating functional phenotypes, as this protein is expressed in most types of microglia as well as recruited monocytes [18]. As previously reported, the metabotropic purinergic receptor P2Y12 is known as a selective marker for the ramified phase of microglia (Fig. 1A) [21–23]. Microglia in P2Y12-/- mice showed dysfunction of directional branch extension toward sites of central nervous system (CNS) injury [21]. P2Y12 expression was dramatically reduced after microglial activation [21]. Extensive loss of P2Y12 immunoreactivity was observed in active cortical lesions of human multiple sclerosis (MS) [22, 24]. Similar to the result in MS, we observed that both AD and tauopathy mice had decreased P2Y12 receptor levels in brain regions with tau pathology (Maeda et al. manuscript in preparation). Notably, a reduction of P2Y12-positive microglia in the cerebral cortex and hippocampus regions occurred in young rTg4510 mice prior to pathological tau accumulation (Maeda et al. manuscript in preparation). *P2Y12* and several other genes were recently identified as unique microglial genes showing homeostatic microglia phenotypes in mouse brain [25]. Therefore, these results indicate that microglia may lose their homeostatic molecular signature and functions before tangle formation. The age-dependent increase of Iba1 immunoreactivity observed in rTg4510 mice may also suggest the conversion from resting phase to active phase of microglial function at early stage of neurodegenerative disease [7]. Nevertheless, further investigations with identification of microglial molecular signatures (microglial gene and microRNA signatures in murine CNS-derived adult microglia were described in [25]) in rTg4510 mice during disease progression will be needed.

P2Y12 RECEPTOR AS AN EARLY-STAGE MARKER FOR MICROGLIAL ACTIVATION

Ramified microglia in the healthy CNS were thought to be immobile due to the low expression of activation-associated molecules [26]. But, these ramified "resting" microglia actually surveyed the environment until any disturbance occurred (reviewed in [27]). Since the housekeeping activity of these surveillant microglia is largely unknown, searching for specific markers of surveillant microglia is essential for understanding the resting phase of microglia. Expression of P2Y12 receptor is remarkable in the resting state while it is significantly reduced after microglial activation [21]. As an early-stage marker, P2Y12 may be a feasible marker for identification of resting microglia. *In vivo* visualization of activated microglia is available with the use of PET tracers of TSPO, although several problems (e.g., non-specific binding, sensitivity for single nucleotide polymorphism) still need to be resolved [28]. Based on our findings, increase of TSPO signal in a tauopathy mouse model was a late event following pathological tau accumulation (Fig. 1C) [7]. Thus, the early phase of microglial activation is hardly detectable by current *in vivo* imaging techniques (Fig. 1A). The targeting of P2Y12 receptor for visualizing resting microglia is being anticipated. P2Y12 receptor is a potent marker for visualizing *in vivo* microglia functions by PET imaging, although positron labeled P2Y12 receptor antagonists have not yet been reported. As potential candidates, reversible competitive antagonists (e.g., cangrelor, ticagrelor) have been synthesized [29]. However, due to their lower lipophilicities and higher molecular weights, these molecules seem to have difficulty crossing the blood brain barrier. For future direction, researchers are looking for novel

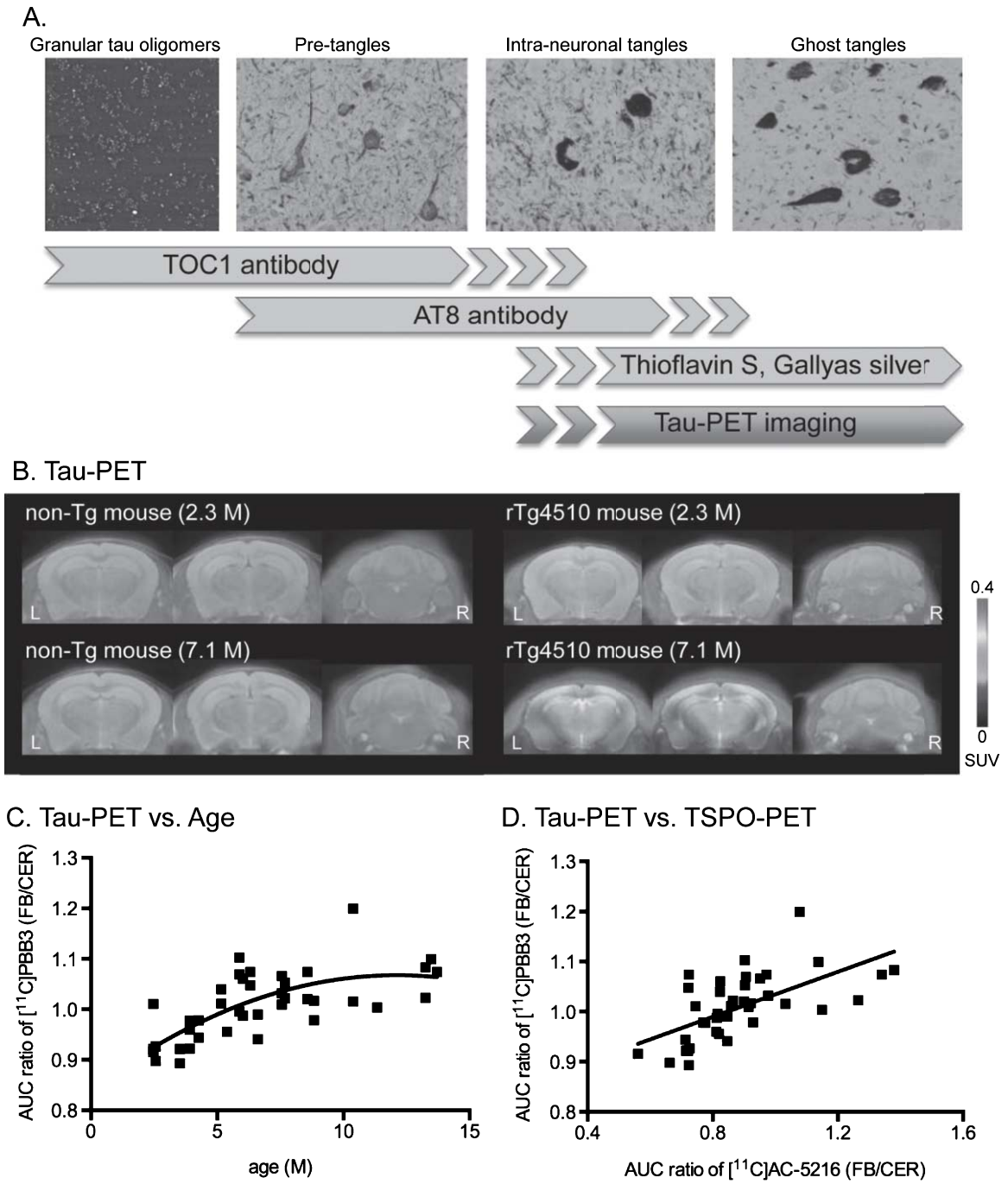

Fig. 2. Characteristics of current tau-PET imaging. A) Process of pathological tau aggregations was determined by conformation, phosphorylation and filamentous structures. TOC1 (tau oligomer specific) antibody and AT8 antibody (mouse monoclonal, Thermo Fisher Scientific) can recognize pre-fibrillar oligomeric tau inclusions, whereas Thioflavin S and Gallyas silver label tangle-shaped tau inclusions. Current tau PET tracers principally recognize filamentous tau aggregates. B) Representative tau-PET imaging with [^{11}C]PBB3 radiotracer in mouse brains. Images of [^{11}C]PBB3 signals in brains of 2.3-month-old non-tg (top left), 7.1-month-old non-tg (bottom left), 2.3-month-old rTg4510 (top right), and 7.1-month-old rTg4510 (bottom right) mice were generated by averaged dynamic scan data. The images showed dorsal hippocampal level of coronal (left), ventral hippocampal level of coronal (middle), and cerebellum level of coronal (right) slices. SUV was calculated by injected dose per tissue volume x body weight. C) Scatterplot of AUC ratio (forebrain to cerebellum) for [^{11}C]PBB3 signals against age (months) of rTg4510 mice ($n = 40$). D) Scatterplot of AUC ratio (forebrain to cerebellum) for [^{11}C]PBB3 signals against AUC ratio (forebrain to cerebellum) for [^{11}C]AC-5216 signals. Spearman's correlation analysis showed significant correlation ($p < 0.05$).

antagonists of P2Y12 receptor with higher lipophilicity and lower molecular weight.

LIMITATIONS OF CURRENT TAU PET IMAGING

In vivo visualization of tau pathology is one of the hot topics for diagnosing neurodegenerative diseases. Recent progress of tau PET imaging has led to successful clinical assessments in patients with tauopathy [30]. Although the off-target binding issue is still under investigation [30], it has become possible to assess the regional distribution and severity of tau pathology. Because current tau PET tracers are designed for targeting filamentous tau aggregates [31–33], these tracers theoretically do not bind with premature tau aggregates (e.g., tau oligomers, pretangles) (Fig. 2A). Our recent study confirmed that micro-PET imaging with [^{11}C]PBB3 (^{11}C-labeled phenyl/pyridinil-butadienyl-benzothiazoles/benzothiazolium 3 [31]) showed an age-dependent increase in [^{11}C]PBB3 signals (Fig. 2B, C) and that [^{11}C]PBB3 signals were positively correlated with TSPO tracer [^{11}C]AC-5216 signals (Fig. 2D; detailed findings were presented in [7]). Since the increase in [^{11}C]PBB3 signals reached a plateau at age 7 months (Fig. 2C), its significant correlation with [^{11}C]AC-5216 disappeared after age 7 months. Notably, TSPO levels were a better indicator for the severity of disease progression at the late stage of tauopathy. On the other hand, our previous finding showed that intracellular tau accumulation labeled by TOC1 antibody (tau oligomer-specific antibody [34]) appeared as early as 1.5 months of age [35]. As the [^{11}C]PBB3 signal increases at 6 months of age, this tracer may not be able to detect tau oligomers (Fig. 2A). Since an early diagnosis of tauopathy is essential, novel tools for detecting pre-fibrillar oligomeric tau inclusions need to be developed.

CONCLUSION

Current observations indicate that reduction of P2Y12-positive microglia appeared at early stage of human MS and tauopathy [22, 24] (Maeda et al. manuscript in preparation). There is accumulating evidence that microglia play important causative roles in neurodegenerative diseases. Imaging biomarkers for neuroinflammation have been developed, targeting specific proteins in activated microglia. As a most popular target for microglial activation, TSPO-PET imaging is now available in human study [28]. Since TSPO is not only overexpressed in activated microglia but also in reactive astrocytes, visualization of other targets for microglial phenotypes is greatly desired. Combinations of tau and microglia imaging will provide novel approaches for enabling an early differential diagnosis of tauopathy.

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Conquering Alzheimer's Disease by Self Treatment

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Abstract. The means are now at hand to conquer Alzheimer's disease (AD). The method is to identify those at risk for the disease before clinical signs develop. That is followed by implementing measures that can effectively prevent disease development. Since biotechnology markers have shown that AD commences at least a decade before cognitive deficits set in, there is an extended window of opportunity to successfully prevent disease development. Methods of identifying those at risk include positron electron microscopy for AD senile plaques, blood or saliva analysis for elevation of the amyloid-β protein fragment terminating at position 42, and cerebrospinal fluid analysis showing a decrease in content of this protein. Of the modalities available, saliva is by far the simplest and least invasive. Once identified, those at risk can prevent disease development through self treatment by consumption of non-steroidal anti-inflammatory drugs, adhering to a Mediterranean diet, and consuming antioxidants such as quercitin which is contained in coffee.

Keywords: $A\beta_{42}$, coffee, Mediterranean diet, non-steroidal anti-inflammatory drugs, saliva

DIMENSIONS OF THE ALZHEIMER'S DISEASE PROBLEM

It would be difficult to overstate the urgency of finding solutions to the Alzheimer's disease (AD) problem. Alzheimer Disease International estimated that there are 35 million people suffering from this disorder at an annual cost of $604 billion. This estimate is contained in the 2010 World Alzheimer Report (http://www.alz.co.uk).

According to the World Health Organization (WHO), AD is the seventh leading cause of death in developed countries. The 2017 United States (US) Alzheimer's Association Report estimates that more than 6 million people just in the US are living with AD. The cost of their care is estimated to be $259 billion, not including unpaid costs of volunteers. There are more than 500,000 US deaths from this cause each year. More ominously, both the WHO and US reports predict that unless effective measures of prevention and treatment AD are discovered, the number of cases may increase two to threefold by 2050.

AD does not affect young people. It is an age specific disorder, increasing dramatically with age in those vulnerable to the disease. Brookmeyer et al. [1] estimated the age-specific incidence rates of AD progressively increases from about 0.17% per year at age 65, to 0.71% at age 75, to 1.0% at age 80, and to 2.92% at age 85. These estimates were based on studies from Boston, Framingham, Rochester, and Baltimore. Such studies indicate that intervention, if it is to be successful, must be started at least a decade before the age of risk for a given individual.

PATHOGENESIS OF AD

AD is characterized by brain deposits of amyloid-β protein terminating at position 42 ($A\beta_{42}$) [2]. It is a relatively insoluble peptide fraction of the amyloid-β protein precursor (AβPP). A more common fraction terminates at position 40 ($A\beta_{40}$). However, this fraction is soluble and is much more readily phagocytosed. It does not accumulate in brain. If $A\beta_{42}$ is

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permitted to accumulate in brain, it produces extracellular deposits in the form of senile plaques. These plaques stimulate an inflammatory response. The inflammatory response, in turn, fully activates the complement system [3]. This results in formation of the membrane attack complex that directly damages residual brain neurons. A progressive loss of these brain neurons occurs, which eventually results in the cognitive deficits that define clinical AD. The age of AD onset varies, presumably because the level of $A\beta_{42}$ production varies. The higher the $A\beta_{42}$ production, the earlier AD onset occurs. A given individual needs to decide when to begin a preventative regimen. This should be 10–15 years prior to the time a relative has come down with the disease.

We reported in 1990 that rheumatoid arthritics, who universally consume anti-inflammatory agents, were relatively spared from AD [4]. This has been confirmed in more than 17 epidemiological studies that have focused on consumption of non-steroidal anti-inflammatory drugs [5].

THERAPEUTIC ATTEMPTS

AD is a graveyard for expensive clinical drug trials. *Chemistry World* in its July 2014 issue reported that in the period between 2000 and 2012, 244 compounds were tested in 413 clinical trials. Only one was approved for use, indicating a failure rate of 99.6%. Even the one approved did not represent an advance. It was for memantine, an NMDA receptor antagonist, which reduces glutamatergic excitotoxicity. It is not an anti-inflammatory agent.

A research strategy that has been aggressively pursued, despite repeated failures, is to administer monoclonal antibodies against epitopes of Aβ. Antibodies are inappropriate because they must be administered parenterally and are not expected to cross the blood-brain barrier. Moreover, they are impractical as a long-term strategy. They would need to be administered parenterally at frequent intervals throughout life.

Nevertheless, enormous resources have been wasted pursuing this doomed-to-fail strategy. Two recent examples are bapineuzemab (Elan/JNJ & Pfizer) [6] and solanezumab (Lilly). Failure of the latter was announced at the 9th Clinical Trials on Alzheimer's Disease meeting in 2016. Both underwent massive Phase III clinical trials which cost hundreds of millions of dollars, and both fell short of meeting their primary efficacy endpoints.

Reasons for this succession of failures include choosing an inappropriate target, choosing an inappropriate way of hitting a target, and commencing treatment too late to rescue cognitive function. The target is $A\beta_{42}$, not $A\beta_{40}$, or some other fraction of AβPP. There are other possible targets based on inhibiting the complement cascade but these have not yet been tested in clinical trials.

EPIDEMIOLOGICAL EVIDENCE OF PREVENTION

Compared with subjects in the lowest Mediterranean diet tertile, subjects in the middle tertile had an AD hazard ratio of 0.85 (95% CI, 0.63–1.16) and those in the highest tertile had a hazard ratio of 0.60 (95% CI, 0.42–0.87) (p for trend = 0.007). In a follow-up analysis, the Mediterranean diet was also associated with a reduced risk of developing mild cognitive impairment and of progression from mild cognitive impairment to AD [7].

METHODS FOR EARLY DIAGNOSIS OF AD AND PREDICTION OF LATER CLINICAL ONSET

Established methods for early diagnosis of AD include positron electron microscopy (PET) for AD senile plaques, blood or saliva analysis for elevation of $A\beta_{42}$, and CSF analysis showing a decrease in content of this protein. These methodologies are expensive to carry out. Their availability is scarce and does not include the general public. The only method that might become available in the future is salivary analysis. It is already in use for DNA determination.

The average person has presently no method available for predicting vulnerability to AD other than family history. The risk of inheritance is 50% if either a parent or a sibling suffers from AD. If both parents suffered from AD, the risk increases to 75%. There will be false positives based on family history varying between 25% and 50%. False negatives are unlikely, since it would require both parents dying before the age of AD onset and all siblings being free of AD.

Saliva tests for $A\beta_{42}$ may provide the widely available predictive test that is needed. Once identified, those at risk can lessen the chance of disease development by consumption of non-steroidal anti-inflammatory drugs, adhering to a Mediterranean diet, and consuming antioxidants such as quercetin which is contained in coffee [8].

False positives, as well as those at risk, can also enjoy benefits of the "Conquering AD" regimen since the benefits go well beyond AD itself. That is because several chronic degenerative disorders have an inflammatory basis. These include age related macular degeneration, Parkinson's disease, frontal temporal dementia, multiple sclerosis, atherosclerosis, and numerous autoimmune disorders [2].

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9913).

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Past to Future: What Animal Models Have Taught Us About Alzheimer's Disease

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Abstract. Alzheimer's disease (AD) impairs memory and causes significant cognitive deficits. The disease course is prolonged, with a poor prognosis, and thus exacts an enormous economic and social burden. Over the past two decades, genetically engineered mouse models have proven indispensable for understanding AD pathogenesis, as well as for discovering new therapeutic targets. Here we highlight significant studies from our laboratory that have helped advance the AD field by elucidating key pathogenic processes operative in AD and exploring a variety of aspects of the disease which may yield novel therapeutic strategies for combatting this burdensome disease.

Keywords: 3xTg-AD, amyloid-β, animal models, comorbidities, inflammation, stem cell therapy, synaptic loss, tau

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia among the elderly, and it is projected that, by 2050, 1 in 3 seniors will develop this insidious disease [1]. Despite immense efforts within academia and the pharmaceutical industry, as of today, there are no effective treatments available [2, 3]. Moreover, the course of the disease is prolonged and the prognostics are poor and a definitive diagnosis of AD is only established when the presence of amyloid plaques and neurofibrillary tangles are confirmed in the postmortem brain from the suspected patient [4].

Neuropathologically, AD is characterized by the abnormal accumulation of extracellular deposits composed primarily of the amyloid-β protein (Aβ), known as plaques, and intracellular aggregates consisting of hyperphosphorylated forms of the microtubule-associated protein, tau, known as neurofibrillary tangles [3]. Aβ is a heterogeneous mixture of peptides ranging from 37 to 43 amino acids in length produced through the sequential cleavage of a type-I membrane-spanning protein known as the amyloid-β protein precursor (AβPP), with 40- and 42-amino acid peptides being the predominant species. AβPP can be cleaved at three different sites, by proteolytic activities referred to as α-, β-, and γ-secretases. Aβ peptides are produced when AβPP is processed first by β-secretase, then by γ-secretase. Cleavage by β-secretase results in the secretion of the large amino (N)-terminal ectodomain of AβPP, known as sAβPPβ, into the extracellular space. The resulting carboxy (C)-terminal fragment is retained in the membrane and subsequently processed by γ-secretase. The vast majority of AβPP is, in fact, processed by an alternative pathway, being cleaved first by α-secretase, resulting in the secretion

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of an N-terminal ectodomain known as sAβPPα, followed by γ-secretase-mediated processing of the membrane-bound C-terminal fragment. Notably, α-secretase activity cuts at a site located *within* the Aβ sequence, thus precluding the creation of Aβ [5].

Once formed, Aβ peptides have a strong tendency to self-aggregate, something that is especially true for the longer species of Aβ like $A\beta_{42}$. Aβ peptides coalesce to form a number of higher-order aggregates characterized by a beta-sheet conformation, including soluble, low-molecular-weight species, including dimers, trimers, and dodecamers, known collectively as oligomers. Aβ can also form a variety of high-molecular-weight aggregates, which are generally insoluble, including protofibrils, fibrils and, ultimately, plaques.

Tau is a microtubule-associated protein that has a role in stabilizing neuronal microtubules and, hence, in regulating axonal transport [6–10]. When released into the extracellular space, tau can modulate the signaling of synaptic receptors and, due to its interactions with scaffolding proteins, tau may also regulate receptors present in postsynaptic sites. Also, recent findings demonstrated that tau is involved in long-term depression in the hippocampus [11, 12]. Altogether, these mechanisms demonstrate a key role of tau in controlling the normal functioning of synapses, which can be severely affected in AD.

For the past few decades, genetically engineered mouse models have been the gold stars of basic AD research and have proven invaluable for understanding how AD pathology develops in the brain and to evaluate and discover new therapeutic targets and disease-modifying strategies. Our research group helped advance our collective understanding of the interrelationship between Aβ and tau pathology in AD by developing a mouse model that develops both amyloid plaques and neurofibrillary tangles. We accomplished this by generating a mouse model that harbors disease-causing mutations in three separate genes, AβPP, tau, and presenilin-1 [13]. Known as the triple-transgenic model of AD, or 3xTg-AD, this approach made it possible not only to investigate the two major pathological hallmarks within the same animal, but also to shed a light into the interaction between Aβ and tau.

In this chapter, we will focus on how our research group has ultimately changed and helped the AD field move forward through the understanding of key pathological mechanisms in AD such as neuroinflammatory processes, synaptic changes, comorbidities associated with AD and stem cell-related research.

NEUROINFLAMMATION: BUILDING UP TO THE STORM

Among the factors associated with aging that reduce the quality of life for the elderly are the alterations that affect the immune system. As we age, the innate immune system becomes dysregulated and is characterized by persistent inflammatory responses [14, 15]. Although inflammation is a fundamental protective response, age-related changes in the immune system can contribute to the increased susceptibility of the elderly to innumerous diseases including AD. More insight into the molecular pathogenesis of the disease is required to better translate basic biological discoveries into safe and effective clinical applications.

We have been particularly interested, over the past few years, in understanding how inflammation impacts Aβ and tau pathology (Fig. 1). Elderly individuals are susceptible to viral and bacterial infections, and these microbial agents could exacerbate the existing inflammatory condition in the brain, accelerating the cognitive decline. It is now well accepted that chronic inflammation mediated by inflammatory receptors such as IL-1R1, Toll-like receptor 4 (TLR4), and tumor necrosis receptor (TNFR) represents a key mechanism by which Aβ drives the development of tau pathology and cognitive decline in AD [16–18]. One important receptor implicated in AD, TLR4, is responsible for detecting microbial products and inducing innate and adaptive immunity [19]. Studies conducted by our group in the 3x-Tg-AD mouse model demonstrated that stimulation of TLR4 by Escherichia coli lipopolysaccharide (LPS) exacerbates tau pathology, via a glycogen synthase kinase-3β (GSK-3β)–dependent mechanism, with chronic inflammation leading to impairments in spatial memory [20]. The activation of TLR4 by pathogen-associated molecular patterns leads to the expression of proinflammatory cytokines, which will then start specific immune responses. Indeed, the brains of 3xTg-AD mice presented significant increased levels of interleukin-1β (IL-1β) after chronic LPS treatment.

There is a growing body of evidence showing that IL-1β turns synaptic plasticity, learning and memory more susceptible to impairment, especially with age [21–23]. Aged animals present specific deficits for long-term potentiation (LTP) [24, 25] and hippocampal-dependent memory [26, 27] after a systemic immune activation, and all of these impairments are blocked by brain infusion of the

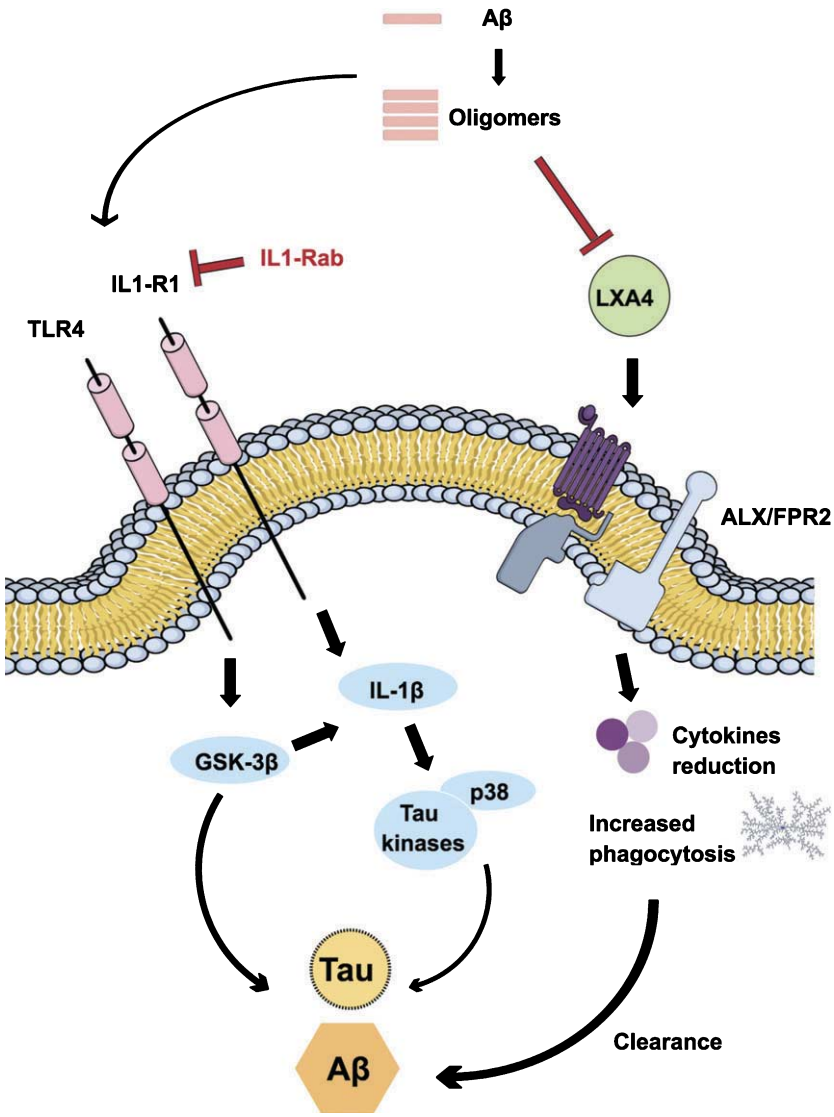

Fig. 1. Inflammatory mechanisms linked to Alzheimer's disease (AD). The activation of inflammatory receptors like IL1-R1 and TLR4 is a key mechanism by which Aβ leads to tau pathology and cognitive decline in AD. Stimulation of TLR4 leads to the expression of proinflammatory cytokines, via a glycogen synthase kinase-3β (GSK-3β)–dependent mechanism, converging on cognitive impairments and pathology progress. Inhibition of IL-1β signaling, by an IL-1R1 antibody, reduces the activation of tau kinases and p38, alleviating cognitive deficits and partly reducing some fibrillar and oligomeric forms of Aβ. During aging and in AD, there is a reduction of lipoxin A4 production, an endogenous pro-resolving mediator. Restoring its levels leads to an alternative activation of microglia, a reduction of overall inflammation, and the promotion of increased phagocytosis and Aβ clearance.

IL-1 receptor antagonist, IL-1ra. In this regard, we demonstrated that inhibition of IL-1 signaling, by chronically treating 3xTg-AD mice with an IL-1R blocking antibody, reduced the activity of several tau kinases in the brain, including cdk5/p25, GSK-3β, and p38-MAPK, also reducing phosphorylated tau levels. Moreover, the treatment significantly altered brain inflammatory responses through the reduction of nuclear factor κB (NF-κB), alleviated cognitive deficits and partly reduced some fibrillar and oligomeric forms of Aβ [17]. Recently, it was demonstrated that IL-1β impairs LTP directly at the synapse and that sensitivity to IL-1β is augmented in aged hippocampal synapses, through an IL-1 receptor subunit reconfiguration [18]. Thus, ours and other studies provide evidence that modulation of IL-1β signaling may offer therapeutic benefit to AD patients, and it has been a con-

stant target of investigation within our research group.

Immune responses need to be tightly regulated in terms of intensity, class, and duration to prevent molecular, cellular, and organ damage. Despite the fact that its neuropathological involvement and consequence in AD still remains to be elucidated, it has been suggested that inflammation plays a dichotomous role in the disease. In young individuals, inflammation is self-limited and resolves by means of an active termination program known as inflammatory resolution [28]. The discovery of this active and highly coordinated process controlled by endogenous pro-resolving mediators modified our understanding of diseases caused by chronic inflammation [29, 30]. In older subjects, however, disturbances in the immune system result in a state of low-grade chronic inflammation. In the brain, persistent and unresolved inflammation has been implicated, with a variable degree of importance, in almost all age-related neurodegenerative disorders. In AD, chronic inflammation, that is characterized by activation of microglia and astrocytes and excessive production of pro-inflammatory mediators, may lead to disease progression and neuronal loss [31–33]. Therefore, new approaches aimed to modulate the inflammatory response in AD might prove efficacious. To this end, we evaluated the role of an endogenous lipid mediator, lipoxin A4 (LXA4), generated during the resolution phase. Through agonistic actions at the G-protein coupled LXA4 receptor ALX/FPR2, lipoxins reduce neutrophil recruitment and activation, leukocyte migration, and cytokine production [34, 35]. In the central nervous system (CNS), LXA4 protects neurons against stroke, the development of neuropathic pain after spinal cord injury [36], and $A\beta_{42}$ toxicity [37]. During aging and in Tg2567 mice, there is a significantly impairment of LXA4 production. Notably, restoration of this mediator signaling led to an alternative activation of microglia, with a reduction of overall inflammation, and the promotion of phagocytosis and Aβ clearance. All these effects were also accompanied by upregulation of synaptic proteins and cognitive improvement [38]. Additionally, aspirin-triggered lipoxin A4 (ATL) also reduced Aβ and phosphorylated tau enhancing the cognitive performance of 3xTg-AD mice [39]. Recently, it was demonstrated the reduction on the levels of LXA4 both in the cerebrospinal fluid (CSF) and hippocampus of AD patients, with a strong correlation with cognitive function [40]. Also, the ability to measure these important mediators in the CSF also provides incentive to explore their potential as diagnostic markers.

Altogether, these data suggest that the inflammatory resolution process is altered by AD, playing a role of great significance in brain homeostasis.

SYNAPTIC LOSS: THE BEGINNING OF THE END

AD is currently an important public health issue, leading to an increased effort over the past years to better understand the causes of it. Several epidemiological studies have demonstrated that synaptic loss has been strongly associated with the cognitive deficits observed in AD. Notably, these impairments are better correlated with the synaptic pathology than either plaques or tangles, therefore suggesting synaptic changes as a central factor for the disease process and progression [8, 13]. Several animal models and clinical studies utilizing familial forms of AD have widely documented the importance of Aβ and tau pathology in the progression of AD.

In this section, we will highlight research findings from our group on how Aβ and tau affects synaptic loss and cognitive deficits in animal models of AD. The idea of Aβ oligomers as toxins responsible for synapse dysfunction and cognitive deficits in AD has aided our understanding of the mechanisms of the disease [41]. However, new evidence has demonstrated that tau also regulates other important processes related to the synaptic function and it is also detected in the dendrites, as well as in pre- and postsynaptic components of normal healthy neurons [42, 43]. Nevertheless, in AD and several other neurodegenerative diseases, known as tauopathies, tau develops post-translational changes that will affect its affinity to microtubules. This process leads to neurofibrillary tangles, which may alter the axonal transport. Moreover, calcium signaling is essential for learning and memory processes; however, its dysregulation may be related to pathological tau changes [6, 7]. Our research group has previously demonstrated that calpain-active cdk5 and ERK1/2 kinases can phosphorylate tau and induce innumerable downstream tau-dependent and independent pathogenic effects, including impairments of synaptic plasticity and cognition [44].

The development of the 3xTg-AD mice by our research group have greatly advanced the AD field, as these mice together promote the development of Aβ and tau pathology and exhibit deficits in synaptic

plasticity, including LTP that occurs before extracellular Aβ deposition and formation of tangles. Such finding demonstrates that synaptic transmission and LTP deficits precedes plaque and tangle formation in the 3xTg-AD mice and implies that synaptic dysfunction is an early manifestation of AD and that extracellular Aβ deposition is not the only factor underlying the synaptic dysfunction [13].

A question that still needs to be addressed in the AD field is how the molecular relationship between Aβ and tau affect the integrity of synaptic function and lead to profound and irreversible cognitive deficits. Bearing this in mind, there are multiple mechanisms by which Aβ and tau can impair synaptic function and lead to severe cognitive deficits (Fig. 2). It has been demonstrated that Aβ promotes tau and its misplaced localization in dendritic projections, and that overexpression of both toxic proteins accelerates synaptic and cognitive impairments [45–47]. Given that Aβ and tau coexist and interact directly between themselves within the synaptic compartment, both proteins may have a synergic role in affecting normal synaptic functions [8]. Our research group has demonstrated that 3xTg-AD mice highlight the importance of intraneuronal soluble Aβ as the initial mediator of tau pathology. Aβ induces tau pathology by altering the levels of the C terminus of heat shock protein 79-interacting protein (CHIP), a known tau ubiquitin ligase responsible for facilitating degradation of hyperphosphorylated tau and caspase-3-cleaved tau [48]. In addition, extracellular Aβ is also involved in the development of tau pathology. As it will be demonstrated in another section of this chapter, studies using induced neuronal-derived pluripotent stem cells (iPSCs) have shown that extracellularly generated Aβ increased tau levels in familial AD neurons and that extracellular Aβ has an important role in tau pathology mediated by inflammation.

Further studies suggest that tau targets the tyrosine kinase Fyn, a member of the Src family, in the postsynaptic density and induces aberrant glutamatergic synaptic transmission via overactivation of NMDARs [49]. Moreover, the reduction in soluble Aβ oligomers is accompanied by a decrease in human tau pathology, including reduced association of tau with PSD-95, and a rescue of learning and memory deficits. Our data therefore indicate that sol-

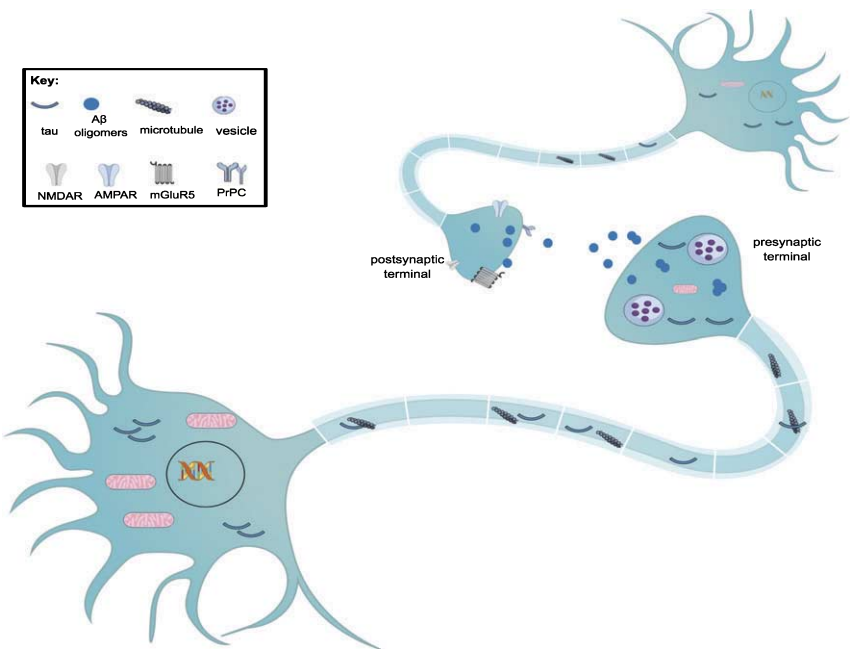

Fig. 2. Formation and mechanisms of synaptic toxicity of tau and Aβ oligomers. During tauopathies, there is a reduction in the number of dendritic spines. Tau does not enter the nucleus of the neuron, resulting in DNA damage. There is a reduction in the number of mitochondria and also in the number of presynaptic vesicles, which leads to synaptic loss. Such loss is also due to the entrance of tau into dendrites and postsynaptic areas. Tau also aggregates extracellularly, enabling it to be captured by other neurons. Aβ oligomers may decrease the number of surface glutamate α-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs); there is a decrease the synaptic strength via an NMDA-dependent pathway. The prion protein-containing oligomer receptor complex (PrPC) interacts with mGluR5, spreading the toxic effect of Aβ oligomers. Moreover, oligomers can interact with a variety of receptors on the pre- and postsynaptic membrane of neurons.

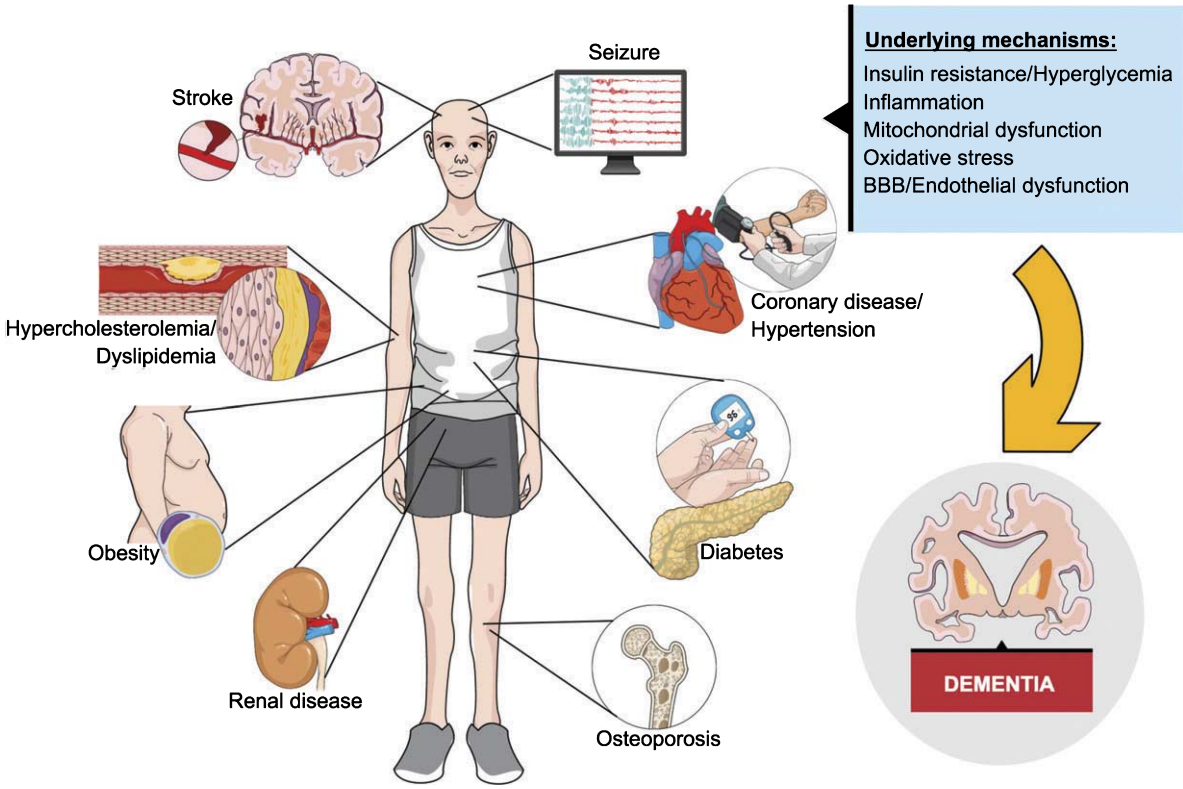

Fig. 3. Comorbidities in Alzheimer's disease (AD). Diabetes, osteoporosis, renal disease, obesity, hypertension and hypercholesterolemia/dyslipidemia, stroke, and seizure are the main comorbidities affecting the onset and progression of AD, adding intricacy to the pathogenesis of the disease. The mechanisms underlying this relationship are assorted and complexes and are highlighted in the blue square, including insulin resistance, inflammation, and oxidative stress. BBB, blood-brain barrier.

uble Aβ, particularly soluble Aβ fibrillar oligomers, facilitate wild-type tau pathology *in vivo* [47].

In summary, these findings highlight the complexity of Aβ and tau relationship and demonstrate how our research group has led to a better understanding of how tau impacts synaptic function and is related to the pathological role of Aβ in synapses.

DIABETES, STRESS, AND AD: THE CHICKEN AND EGG QUESTION

Despite intensive research efforts over the past few decades, the mechanisms underlying the etiology of sporadic AD (sAD), which represents the most common form of the disease, remains unknown. This is due, at least in part, to the fact that the majority of sAD patients are elder subjects that commonly suffer from a variety of co-morbidities (e.g., stroke, stress, diabetes, seizures, osteoporosis, and renal disease). On average, people living with dementia who are over 65 years old have four comorbidities (Fig. 3). These comorbidities add complexity to the pathogenesis of sAD, affecting its onset and progression [50, 51]. Over the past decade, multiple studies have been performed in animal models to understand the impact of these co-morbid medical conditions on AD pathogenesis [52, 53]. Here, we describe the most relevant studies in the last years and those in which our research group has been working on.

Among the variety of co-morbidities one of the most prevailing conditions is diabetes (Fig. 4). Interestingly, recent epidemiological studies indicate that diabetes significantly increases the risk of developing AD, suggesting that diabetes may play a causative role in the development of AD pathogenesis [54]. Moreover, AD and diabetes share several clinical and biochemical features, suggesting common molecular pathways underlying these two diseases [55–58]. The presence of insulin receptors (IRs) in the brain provides important evidence that the brain is a target organ for insulin. Specifically, IRs in the CNS are highly expressed in cognition-related regions, indicating that insulin signaling influence memory, neural plasticity, and cognition [59–66]. Recent evi-

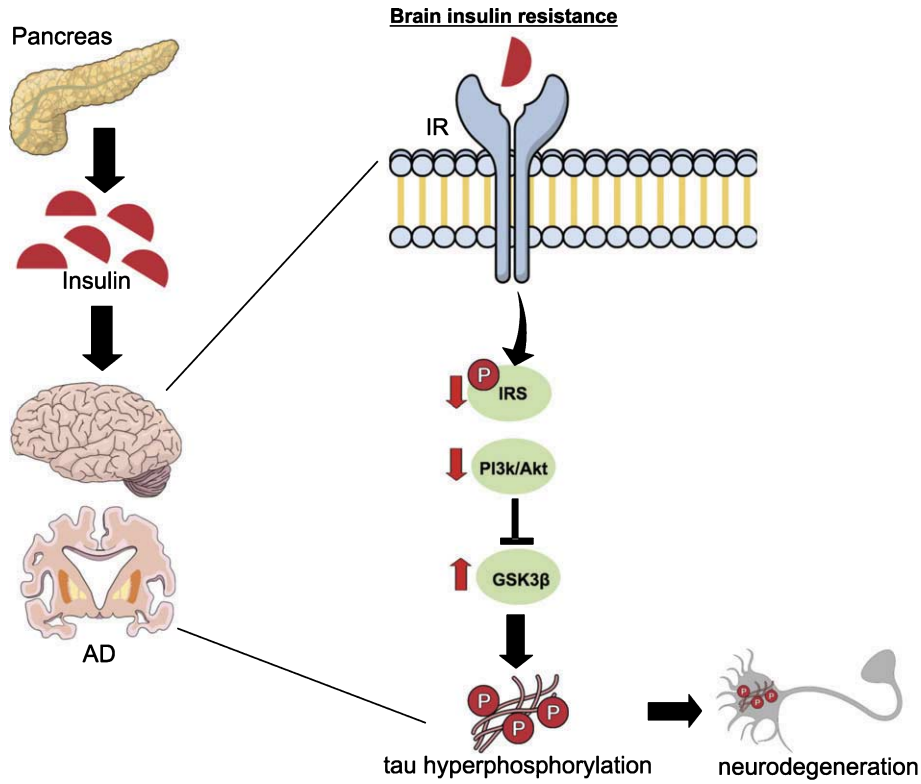

Fig. 4. The role of impaired brain insulin signaling in tau pathology. Disturbance of brain insulin signaling has been suggested to be a key causative event underlying sporadic AD pathogenesis. In type 1 and type 2 diabetes, insulin deficiency and resistance, respectively, lead to an altered insulin signaling pathway in brain tissue. Impaired insulin/insulin receptor signaling leads to decreased insulin-mediated activation of PI3k/Akt signaling activity, resulting dephosphorylation (activation) of GSK-3β. Consequently, GSK-3β activation directly promotes tau hyperphosphorylation and formation of neurofibrillary tangles. Brain insulin signaling dysfunction culminates, then, in synaptic failure and memory decline. IR, insulin receptor; IRS, insulin-receptor substrate; PI3k/Akt, phosphatidylinositol 3 kinase/protein kinase B; GSK-3β, glycogen synthase kinase 3β.

dence reveals that aberrant brain insulin signaling contributes to the pathogenesis of AD [67, 68], and brain insulin resistance is an early common feature of AD [62, 69, 70]. Interestingly, data obtained from human [71] and animal models have shown that diabetes could induce Aβ pathology [72, 73] and promote aberrant tau modifications [74–76]. However, the underlying molecular mechanisms connecting these two disorders are still not well understood. Elucidating these mechanisms is crucial because the number of diabetic and AD patients is expected to increase exponentially in the next decades.

Specifically, our group has focused on understanding how diabetes can alter tau pathology and affect the cognitive and synaptic function. Interestingly, several preclinical studies have shown that modeling type 1 (T1D) or type 2 (T2D) diabetes in rodents results in an increase in tau phosphorylation versus normal controls animals [52, 77]. Using streptozotocin (STZ) treatment, a glucosamine-nitrosourea compound that is toxic to the insulin-producing β-cells of the pancreas inducing hyperglycemia and insulin deficiency in mice, rendering them a valuable model to study T1D [78], we have demonstrated that depletion of endogenous tau mitigates behavioral and synaptic deficits induced in T1D-like mice [52]. In this sense, although induction of T1D in non- transgenic (Ntg) mice led to cellular and behavioral deficits, it did not do so in tau- knockout (tauKO) mice. We showed that STZ treatment causes hyperphosphorylation of tau in Ntg mice through activation of GSK-3β. These increments on hyperphosphorylated tau correlate with spatial cognitive deficits and changes in synaptic proteins. Notably, tauKO mice treated with STZ show no cognitive or synaptic deficits. Overall, our data indicate that T1D impairs cognition via tau-dependent mechanisms, and genetic deletion of endogenous tau gene prevents the synaptic degeneration and cognitive impairment. Hence, these data indicate that tau proteins are crucial downstream targets of the insulin

pathway and mediators of cognitive deficits in a condition of insulin deficiency, representing a potential therapeutic target for patients with diabetes and AD. We are now investigating the role of tau mediating the cognitive/synaptic deficits in T2D, which represents the most common form of the disease.

Current epidemiological evidence indicates that life experiences, including chronic stress, are a risk for AD [79, 80]. In fact, hypothalamic-pituitary-adrenal axis dysfunction as well as elevated levels of cortisol in plasma and CSF are found in AD patients [81], and multiple key studies indicate that stress modulates synaptic plasticity and memory processes [82, 83]. Furthermore, recent studies in animal models have found that stress and stress hormones, including glucocorticoids and corticotrophin-releasing hormone, play a crucial role in AD pathogenesis by modulating Aβ production and degradation [84–86], and impairs tau pathology by modulating key kinases involved in tau phosphorylation or by mislocalizing tau protein to the somatodendritic compartment [85, 87, 88]. Together, these findings suggest that stress and several stress mediators play key roles in modulating AD pathogenesis.

Our group has investigated the impact of short-term, multi-modal modern-life like stress, which often last for hours, on AD progression and its implication in synaptic plasticity and cognitive function. Several lines of evidence support the importance of stress duration and modalities on cognitive function [82, 83, 89]. This matter is extremely important, because modern-life stress often involves multiple concurrent psychological, social, and physical stresses [90]. Therefore, it is fundamental to elucidate the effect of multiple concurrent stresses on the onset and progress of AD pathogenesis. We found that short-term multimodal stress, lasting for 5 hours, severely reduced the number of the spines in 3xTg-AD mice. In addition, this form of stress increased Aβ oligomers by modulation of AβPP processing via upregulation of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1) steady state levels without altering Aβ degradation. This increase of Aβ oligomers might impact the synaptic plasticity and induce robust synaptic loss in the 3xTg-AD mice [53]. Overall, our data suggest that short-term, complex (multimodal) stress, recapitulating salient features of modern-life conditions, is a key factor that triggers AD pathogenesis and severely affects memory and synaptic plasticity in 3xTg-AD mice.

In agreement with these results, we sought to evaluate if blocking the effects of glucocorticoids could help reduce pathology and cognitive decline in 3xTg-AD mice. With this purpose, we used the glucocorticoid receptor antagonist mifepristone (RU486). Mifepristone treatment leads to robust reductions in Aβ levels and plaques through the induction of a 17 kDa cleavage of AβPP, and reduces tau hyperphosphorylation via reduction in p25 levels [91]. Hence, our results show that compounds targeting the glucocorticoid system could be useful for the treatment of AD. However, further studies will be necessary to determine the long-lasting effect of this short-term multimodal stress event in AD pathogenesis.

Owing to the rapid growth in the number of both diabetic and AD patients, and the current impact of a stressful modern life, identifying the clinical associations between those disorders and elucidating the molecular mechanism that mediate their associations could provide protection from the profound medical and economic impact that AD will have over the ensuing decades.

STEM CELL THERAPY IN AD: BACK TO THE FUTURE

The timing for the development of therapeutic strategies that turn in real opportunities for AD patients is really critical, especially due to the lack of effective drugs to cure AD. Currently there are over 100 trials and about 80 drugs in the pipeline, and 99.6% of clinical trials have failed to translate into approved treatments [92, 93]. These disappointing results have encouraged an increased focus on the development of alternative novel and innovative methods. Over the past decade, the potential use of stem cells to treat neurodegenerative diseases, such as AD, Parkinson's disease, and amyotrophic lateral sclerosis have received more attention because of its promising capacity as a regenerative and replacement therapy. With these lines, multiple different studies have shown that using murine neural stem cells have provided compelling evidence of their beneficial effects in motor and cognitive function after different models of brain injuries [94–96], thus the use of stem cell therapy may be a potential treatment for neurodegenerative diseases such as AD [97].

We have conducted pioneer preclinical studies in the 3xTg-AD mice, which develop amyloid plaques, tangles, and important synaptic and cognitive deficits [97], to determine whether neuronal stem cells (NSC)

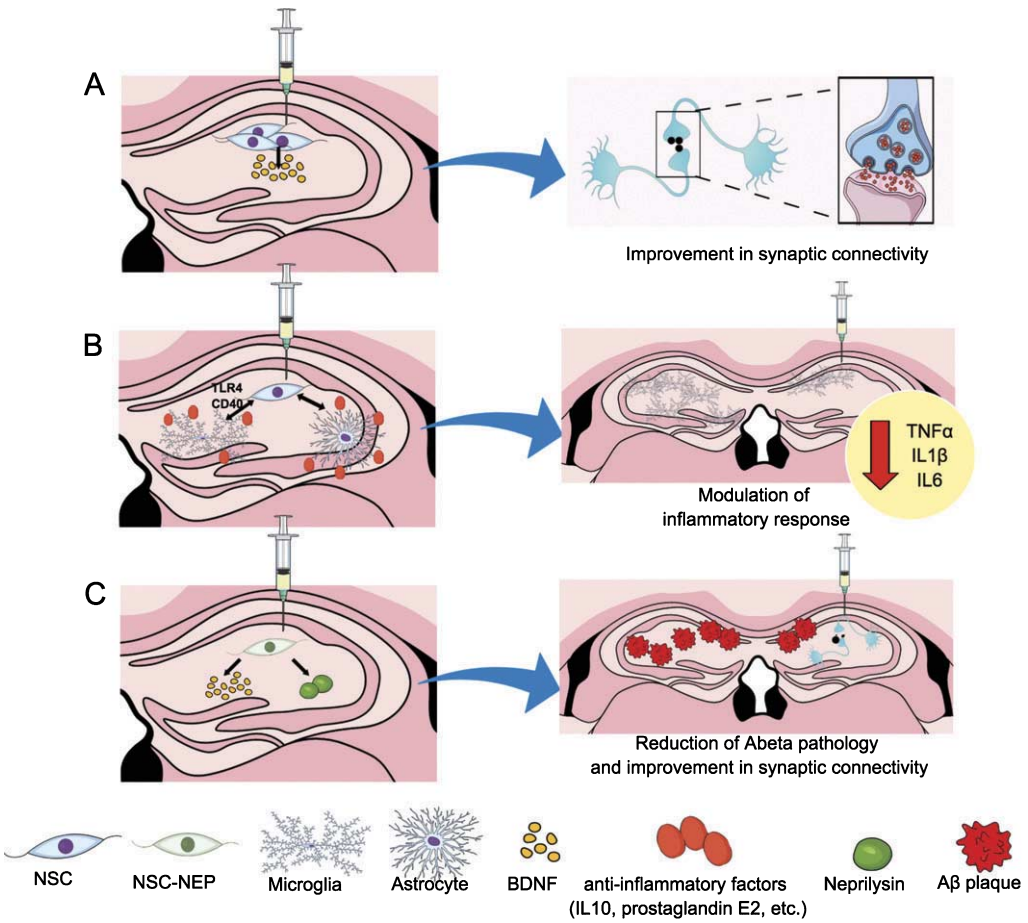

Fig. 5. Underlying mechanisms to potential stem cells therapeutic effects. A) Hippocampal neural stem cells injection lead to an increase in BDNF production and a restoration of cognitive and synaptic deficits in 3xTg-AD mice. B) Stem cells can exhibit anti-inflammatory properties interacting with microglia and astrocytes. Among these effects, NSC might reduce microgliosis and the expression of proinflammatory cytokines such as TNFα, IL1β, or IL6, through CD40 or toll-like receptor 4 (TLR4) signaling pathways. C) The hippocampal injection of NSCs delivering neprilysin lead to a reduction in Aβ pathology in addition to the improvement in synaptic connectivity described in A. NSC, neural stem cell; BDNF, brain-derived neurotrophic factor; CD, cluster of differentiation; TNFα, tumor necrosis factor alpha.

transplantation may offer symptomatic or disease-modifying effects in AD (Fig. 5). Our laboratory demonstrated for the first time that bilateral transplantation of mouse NSC in aged 3xTg-AD mice restored cognitive and synaptic deficits without modifying either plaques or tangle pathology. Among the possible molecular mechanisms underlying these benefits, we found that NSCs produces high levels of brain derived neurotrophic factor (BDNF) and a reduction of BDNF via shRNA-mediated mechanism prevent the cognitive benefit and reduces the effect in the synaptic density [98]. Similar findings were observed in a following study using a different AD transgenic model, the AβPP/PS. In this model, the restoration of both cognitive and synaptic deficits was associated with elevated levels of BDNF and its receptor TrkB. Interestingly, they also found that NSCs treatment did not affect Aβ pathology in APP/PS1 mice [99]. Therefore, these compelling preclinical findings suggest that this therapeutic approach may provide important benefits in patients with advanced existing pathology via improving multiple cognitive-related proteins.

However, for a successfully transition of stem cell-based approach into a clinical application, a suitable human stem cell line is necessary to be identified and tested in preclinical AD models in order to assess its efficacy and safety. Along with this idea we have used a human CNS stem cell line (HuCNS-SC) derived from fetal brain tissue to determine whether cognitive impairment could be restored in two relevant models of AD that exhibit either Aβ and tau

pathology (3xTg-AD) and extensive neuronal loss (CaM/Tet-DT_A). Our study demonstrated a robust therapeutic efficacy of clinically relevant human CNS stem cells in these two complementary models of AD [100]. Specifically, we observed that HuCNS-SC cells recover the cognitive function in both 3xTg-AD and CaM/Tet-DT_A models via improving the synaptic connectivity as evidenced by an increase of synaptic levels and growth-associated proteins. Interestingly, our study also revealed that HuCNS-SC transplantation has no effect on Aβ and tau pathology suggesting that the mechanism of action occurs downstream from these pathologies and probably in a similar way to our previous study by using allogeneic murine NSCs, since HuCNS-SC also produces high levels of the neurotrophin BDNF [98]. Overall, our findings suggest that the mechanisms by which NSCs treatment improve AD cognitive symptoms is mediated via neuroprotection and trophic support rather than neuronal replacement, although we cannot discard that other possible mechanisms can take place. For example, certain stem cell population exhibits robust anti-inflammatory properties. In particular, several studies have shown important anti- inflammatory effect of mesenchymal stem cells through the production of anti- inflammatory mediators such as interleukin-10 and prostaglandin E2, or via stimulation of microglial phagocytosis or microglia production of the Aβ-degrading enzyme neprilysin and also by modulation of CD40 signaling [101–105]. Likewise, the effect of NSC in the immune system is currently under intensive research and new evidence suggests that NSCs could reduce microgliosis and the expression of proinflammatory cytokines such as tumor necrosis factor-α [106]. Another mechanism is via suppression of glial and TLR4 activation and its downstream signaling pathways [107]. Although these studies suggest an important role of stem cell in the modulation of the inflammatory response further studies remain necessary to determine the molecular mechanisms by which stem cell transplantation modulate inflammation in AD pathology. Moreover, another aspect to clarify is to determine if stem cell transplantation alters inflammation directly or simply as a result of tissue injury or xenotransplantation-associated artifacts.

Previously, we have indicated that NSCs can improve cognitive defects in an AD preclinical model through the improvement of synaptic connectivity, although they appear to have no effect on Aβ or tau pathology [98, 100]. Given the complex nature of this disease and the multiple pathways and regions affected, a single small molecule approach may not provide substantial benefit, and the NSC benefits may loss efficacy as pathology continues to develop. Therefore, a combinatory intervention may be a more realistic approach to treat AD patients. For example, supplementing NSC transplantation with Aβ and/or tau-targeting therapies could provide additional long-term benefits. In addition, NSCs could themselves be used to deliver therapeutic proteins due to its capacity to migrate throughout the brain and localize to areas of brain pathology [96, 108]. In this regard, we have tested whether NSCs that deliver disease-modifying proteins such as the Aβ-degrading enzyme, neprilysin (NEP) could provide more effective means. Our findings critically demonstrated that sNEP-expressing NSCs survive for a long period of time and secrete sNEP leading to a markedly reduction of Aβ pathology and enhancing the synaptic connectivity in two transgenic AD models (3xTgAD and Thy1-APP transgenic mice) [109]. Thus, sNEP-expressing NSCs represent a promising therapeutic approach that combines the neurotrophic-mediated benefits of stem cell transplantation with the widespread delivery of a disease-modifying protein and further studies will be needed to determine whether such approach can be translated to an eventual clinical application.

CONCLUDING REMARKS

We discussed in this chapter findings from our laboratory that illustrate critical factors to initiate AD pathology, co-morbidities that contribute to disease progression and cognitive decline, and potential cell-based treatments. The majority of our understanding on AD mechanisms has come from transgenic mice such as the 3xTg-AD model; however, improved models should be created, especially focusing on sporadic AD, in order to maximize the discovery and development of new therapies. Now more than ever, it is crucial to understand the exact pathological mechanisms of disease progression, with the broader purpose of sharply reducing the number of people suffering and dying from AD.

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Iron and Alzheimer's Disease: An Update on Emerging Mechanisms

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Abstract. Iron is a crucial transition metal for life and is the most abundant transition metal in the brain. However, iron's biological utility as an effective redox cycling metal also endows it with the potential to catalyze production of noxious free radicals. This "Janus-faced" nature of iron demands a tight regulation of cellular its metabolism. This regulation is crucial in the CNS, where iron plays myriad keystone roles in CNS processes, including mitochondrial energy transduction, enzyme catalysis, mitochondrial function, myelination, neurotransmitter anabolism and catabolism. Aberrations in brain iron homeostasis can elevate levels of this redox-active metal, leading to mislocalization of the metal and catastrophic oxidative damage to sensitive cellular and subcellular structures. Iron dyshomeostasis has been strongly linked to the pathogenesis of Alzheimer's disease (AD), as well as other major neurodegenerative diseases. Despite the growing societal burden of AD, no disease-modifying therapy exists, necessitating continued investment into both drug-development and the fundamental science investigating the disease-causing mechanisms. Targeting iron dyshomeostasis in the brain represents a rational approach to treat the underlying disease. Here we provide an update on known and emerging iron-associated mechanisms involved in AD. We conclude with an overview of evidence suggesting that, in addition to apoptosis, neuronal loss in AD involves "ferroptosis", a newly discovered iron- and lipid-peroxidation-dependent form of regulated necrosis. The ferroptosis field is rapidly progressing and may provide key insights for future drug-development with disease-modifying potential in AD.

Keywords: Alzheimer's disease, amyloid-β, amyloid-β protein precursor, apoptosis, astrocytes, ferroptosis, iron, lipid peroxidation, oxidative stress, neuroinflammation

INTRODUCTION

As the global population ages, enormous resources will be needed to provide adequate care for the growing number of individuals afflicted by Alzheimer's disease (AD) [1]: the most common cause of dementia, which accounts for up to 80% of all documented cases [2]. AD is an insidious and progressive neurodegenerative disorder, involving substantive cortical and hippocampal neuronal loss [3] that progresses for 20–30 years before clinical onset [4]. Despite the staggering and increasing socioeconomic burden of AD, with >100 million cases predicted by 2050 [1], no disease-modifying therapies are yet available to effectively treat this disease.

The disease is characterized by brain atrophy, extracellular deposition of amyloid-β (Aβ) peptide in senile plaques, the intraneuronal accumulation of hyperphosphorylated tau, neuronal and synaptic loss, chronic inflammation, and oxidative stress [5–10]. Moreover, despite being the focus of decades of intense research, the cause of AD, especially sporadic AD, is elusive. Although the greatest risk factor for AD is aging [11], the pathophysiological mechanisms underlying the role of aging in the development AD are poorly understood.

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Based on the hallmarks of Aβ plaques and neurofibrillary tangles of hyperphosphorylated tau, the "amyloid" and "tau" hypotheses have dominated research into AD etiology. While prevailing drug-development paradigms are predicated on these hypotheses, thus far, effective disease-modifying treatment options remain elusive [3]. This scenario strongly indicates the need to forge new biological models of AD, particularly those that address the advances in our understanding of the underlying etiology of AD-associated neurodegeneration. This approach will be the first step in implementing drug-development strategies that demonstrate disease-modifying activity.

Unlike familial AD, which accounts for <1% cases and is associated with genetic mutations in key proteins and enzymes (e.g., presenilins) associated with amyloid-β protein precursor (AβPP) processing, the initiating event responsible for onset of sporadic AD, particularly early in the prodromal phase of the disease remains masked by uncertain downstream events.

The incidence of AD and oxidative damage to the brain increases with age [11]. Moreover, there is an overwhelming body of evidence that oxidative stress fundamentally contributes to AD pathophysiology and similarly increases with age [6, 7, 11, 12]. Importantly, the dysregulation of redox-active metals (e.g., iron) within the brain appears to underpin the generation and pathological progression of brain oxidative-stress [3, 13].

Accumulating evidence indicates that AD is closely associated with the cumulative effects of oxidative stress, much of which can be linked to iron, within the brain [8, 14, 15]. Indeed, levels of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [16] and lipid hydroperoxides (LOOH) [17], are significantly higher in AD than in healthy control brains. This increase in ROS, as well as their redox-active degradation products, can, at least in part, be attributed to a pathological increase in the levels of redox-active metal ions, particularly iron and copper [13, 18]. Importantly, oxidative stress potentiates the neurotoxic oligomerization of Aβ and tau tangles [3], activation or senescence of astrocytes and microglia, which collectively promote neuroinflammation, and glutamate-induced neurotoxicity (e.g., excitotoxicity and a newly described iron-dependent form of cell death termed "ferroptosis") [19, 20], all of which ultimately lead to neuronal demise [3, 15]. Therefore, increased knowledge regarding the causes and downstream targets of iron-induced oxidative stress in AD will allow us to forge the way to new and lateral therapeutic targets with disease-modifying activity.

In AD, there is pathological accumulation of iron in the hippocampus and cerebral cortex that co-localizes with classical AD lesions, such as extracellular senile plaques of aggregated Aβ, and intracellular tangles of hyperphosphorylated tau [3, 4]. Iron is considered a central player in oxidative stress in AD because, as a redox-active transition metal, it can cycle between Fe(II) and Fe(III) states in biological systems [21, 22]. While this behavior is responsible for the immense biological utility of iron, when dysregulated, it can drive the formation of highly damaging hydroxyl radicals (•OH), and lipid alkoxyl (LO•) and peroxyl (LOO•) radicals, the catalysis of which involves Fe(II)-induced cleavage of H_2O_2 [15], or LOOH [17, 20, 23], respectively, via Fenton- and Haber-Weiss-chemistry.

While the downstream iron-dependent effects of H_2O_2 cleavage are apparent, the precise and quantitatively dominant sources of H_2O_2 in AD are unclear. The hallmark AD lesions (e.g., Aβ aggregates and neurofibrillary tangles of tau) are thought to contribute to production H_2O_2, but strong evidence indicates that significant quantities of H_2O_2 also originate from other sources (e.g., dysfunctional mitochondria, and other cellular oxidases such as H_2O_2-producing monoamine and polyamine oxidases, and inflammatory cytokine-activated NADPH oxidases) [15]. Importantly, increasing evidence suggests radical-mediated oxidation of biological substrates (e.g., membrane lipids) is a key feature of AD pathogenesis [23].

The remainder of this review will provide an update on known and emerging roles of iron in the pathogenesis of AD, as well as the aspects of iron biology in the CNS that are relevant to understanding these roles.

IRON AND OXIDATIVE STRESS: KEY REACTIONS AND CONCEPTS

Iron can act as a pro-oxidant by catalyzing the formation of ROS [21]. The classical pro-oxidant reaction of iron, the Fenton reaction [24], results in the formation of highly-reactive •OH from H_2O_2, according to the equation:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \quad (\mathbf{Reaction1})$$

The one-electron reduction of dioxygen by Fe^{2+} can also generate superoxide anions ($O_2^{\bullet -}$) according to the following reaction:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -} \ (\mathbf{Reaction2})$$

These superoxide anions can then be dismutated, either enzymatically by superoxide dismutases (SODs) or non-enzymatically, to yield H_2O_2, according to the following reaction:

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \ (\mathbf{Reaction3})$$

Additionally, or if the activity of SOD activity is rate-limiting, superoxide anions can reduce trace amounts of labile aqueous Fe^{3+} to form dioxygen and regenerate Fe^{2+}:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \ (\mathbf{Reaction4})$$

The sum of Reaction 1 (Fenton reaction) and Reaction 4 is commonly known as the Haber-Weiss reaction, with iron as the catalyst [24]:

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + HO^- \ (\mathbf{Reaction5})$$

The Haber-Weiss reaction illustrates that in the presence of catalytic amounts of redox-active aqueous low-M_r iron, which increase in peripheral tissues under conditions of iron overload, or in the brain in various CNS pathologies (for reviews, see [3, 13, 18, 25–27]), H_2O_2 may provide a ready source of damaging •OH in the presence of a ferrireductants such as superoxide that regenerates the reduced form of the metal. Importantly, other abundant cellular reductants (e.g., ascorbate, α-tocopherol, and GSH) can also reduce ferric ions in an analogous manner to superoxide in Reaction 4 [28–30]. Thus, cellular reductants such as ascorbate and GSH, which typically function in an anti-oxidative capacity when present at normal physiological levels, can have pro-oxidant activities in the presence of catalytic concentrations of labile iron. Importantly, the iron-catalyzed formation of highly reactive and damaging ROS such as •OH, or lipid alkoxyl radicals (in the case of lipid peroxidation), is primarily responsible for the ability of labile iron to cause oxidative stress. In the case of AD, and other CNS diseases associated with increased concentrations of redox-active or labile iron, these iron-driven Haber-Weiss-like reactions underpin much of the oxidative pathology.

IRON TRAFFICKING, STORAGE, AND UTILIZATION: AN OVERVIEW

Iron is essential for the survival of all cells, as demonstrated by cell death following excessive iron depletion [21, 31, 32]. However, too much iron within cells, tissues, and organs invariably leads to oxidative damage to key macromolecules, including DNA, RNA, proteins, and lipids [33]. Adult humans contain 3-5 g of iron, of which 70-80% is found within erythrocyte hemoglobin, 10-20% is stored within macrophages and hepatocytes, and 3-4% is within heme-bound myoglobin [21, 22]. Within nucleated cells, most iron storage typically occurs within ferritin nanocages [34]. The remainder of the iron is present in other heme-containing proteins (e.g., cytochromes), iron–sulfur cluster (ISC)-containing proteins (e.g., succinate dehydrogenase) [35, 36] and non-heme/non-ISC iron-containing proteins (e.g., 2-oxoglutarate-dependent dioxygenases, BH_4-dependent tyrosine, tryptophan and phenyalanine hydroxylases, as well as the lipoxygenases) [37, 38].

As discussed further below, improperly sequestered iron tends to catalyze the production of toxic ROS through Fenton and Haber-Weiss-type reactions [21]. At the organismal level, iron homeostasis is controlled only through the regulation of iron uptake, as there is no regulated means of the body "ridding" itself of excess iron [21]. In contrast, at the cellular level, iron homeostasis is tightly controlled at several levels, including the import, storage and efflux of iron [21, 39–41].

Iron uptake routes: Transferrin and non-transferrin iron

In terms of the uptake of iron, two major categories of iron-import exist: transferrin (Tf) and non-Tf iron uptake. Under physiological circumstances, particularly in peripheral tissues, virtually all cells favor the import of Tf-bound iron, which is internalized by receptor-mediated endocytosis after binding to Tf receptor 1 (TfR1) [40, 41]. Ferric iron is released from Tf within the endosome after its acidification and is then reduced by an endosomal ferrireductase (e.g., six transmembrane epithelial antigen of the prostate 3 [STEAP3] [42]) [41], or by a novel mechanism involving cellular ascorbate [43–45]. Following the reduction of ferric iron within the endosomal lumen, the resulting ferrous iron is transported across the endosomal membrane by divalent metal transporter 1 (DMT1) in a proton-coupled manner [46],

or in some cases, ZRT/IRT-like protein may also be involved [47]. The protein poly(rC)-binding protein 2 (PCBP2), which is an RNA-binding protein that has been shown to function as an intracellular iron chaperone that delivers iron to ferritin and at least some non-heme-iron enzymes, was recently identified as a DMT1-binding partner that regulates iron influx from Tf across the endosomal membrane to the cytosol [48]. As PCBP2 binds iron and associates with ferritin to deliver iron [37, 49], this may be the first direct evidence of an iron-transport metabolon.

Under physiological conditions, almost all iron in the circulation is bound to Tf, although saturation of Tf with iron is normally ~30% [21]. In diseases resulting in the excessive loading of tissues with iron, Tf becomes saturated with iron, with excess plasma iron occurring as non-Tf iron [21]. The exact uptake route(s) for non-Tf iron remains unclear but are known to involve one or more cell surface ferrireductases (e.g., duodenal cytochrome *b*, DCYTB [50]) or the release of cellular reductants, such as ascorbate [43, 51–53]. These complementary ferrireduction mechanisms reduce ferric non-Tf iron to its ferrous state that can then be imported by transporters such as the transmembrane protein, DMT1 [46], or the ZIPs, ZIP14 or ZIP8 [47].

In Tf- and non-Tf iron uptake, the iron that has entered the cytosol becomes part of a functionally-characterized chelatable or labile iron pool (LIP), which can be utilized for metabolism (e.g., production of iron-containing proteins and enzymes, stored in ferritin or released back to the extracellular space. As such, iron that enters the transitory LIP is either: 1) stored in ferritin; 2) utilized by downstream metabolic pathways (e.g., imported into mitochondria for usage in ISC and heme synthesis, and/or incorporated in cytoplasmic iron-requiring proteins); or 3) released from the cell by the ferrous iron exporter, ferroportin [40, 41].

In non-erythroid cells, including brain cells, the majority (i.e., 70–80%) of this nascently imported iron is thought to be incorporated into ferritin [34]. Ferritin is a multimeric protein composed of 24 subunits that forms a hollow sphere capable of storing ~4,500 iron atoms as a mineralized ferric, phosphate, and hydroxide (ferrihydrite) core [34, 54, 55]. In mammals, there are two ferritin subunits: H-ferritin (heavy subunit, encoded by *FTH1*) and L-ferritin (light subunit, encoded by as *FTL*), which hetero-polymerize to form different "isoferritins" with tissue-specific distributions [34, 54, 55]. Ferrous iron that is bound by ferritin is first oxidized to ferric iron by the ferroxidase activity of H-ferritin in an oxygen-dependent manner [34, 54, 55]. Subsequently, ferric iron core formation commences at carboxyl groups on glutamates of L-ferritin, which is devoid of ferroxidase activity [34, 54, 55]. This enclosure and sequestration of iron as ferrihydrite is vital, as it maintains iron in a redox-inert state [34, 54, 55].

Importantly, ferritin will release iron in a tightly-controlled manner under *in vivo* conditions by targeted autolysosomal proteolysis of the ferritin nanocage, although proteasomal degradation of the protein can occur under specific conditions of therapeutic relevance in which iron chelators are used [34, 56]. The targeting of ferritin for autophagic turnover (i.e., ferritinophagy) has recently been shown to involve nuclear receptor coactivator 4 (NCOA4), which binds to autophagy-related protein 8 (ATG8) proteins on newly formed autophagolysosomes and recruits ferritin as a cargo molecule [57].

Regulation of cellular iron levels: Post-transcriptional control

Due to iron's ability to promote oxidative stress, cellular iron levels and processing are tightly controlled. One major mechanism by which cellular iron homeostasis is controlled is by a post-transcriptional mechanism that modulates the synthesis of key iron metabolism proteins (e.g., TfR1, ferritin, ferroportin, and AβPP) that are involved in iron uptake, storage and release [31, 39]. Specifically, the iron regulatory protein (IRP)-iron responsive element (IRE) system is responsible for this mode of regulation and allows for rapid changes in the translation of key iron metabolism proteins in response to changing intracellular iron levels [31, 39, 58]. This system depends on the mRNA-binding proteins, IRPs-1 and -2, which post-transcriptionally control the expression of mRNAs possessing IREs [31, 39, 58]. IRPs bind to IREs in the 5′- or 3′-untranslated regions (UTRs) of key mRNAs involved in iron metabolism with high affinity in iron-depleted cells, either suppressing the translation of the mRNA (i.e., mRNAs in which the IRE is located in the 5′-UTR; e.g., *FTH1, FTL, ferroportin*, and *AβPP*), or by enhancing mRNA stability against nuclease attack (i.e., mRNAs in which the IRE is located in the 3′-UTR; e.g., TfR1, DMT1-I, etc.) [39, 41].

Under conditions of increased cellular iron, which can be potentiated by endogenous reductants such as ascorbate [43], IRP1 loses its IRE-binding activity by acquiring an ISC (4Fe-4S cluster) [58]. The

acquisition of this 4Fe-4S cluster converts IRP1 into a cytosolic aconitase. In the case of IRP2, iron-dependent, proteasomal degradation is the major regulatory mechanism [59].

REGULATION OF BRAIN IRON

Transport and trafficking of iron in the brain

In contrast to cells in the periphery that engage almost exclusively in Tf-bound iron uptake under physiological conditions, different types of brain cells appear to be adapted either for the uptake of Tf-bound iron (e.g., neurons) or non-Tf-bound iron (e.g., astrocytes, oligodendrocytes, and microglia) [60–62]. The majority of brain iron derives from the Tf-iron in the blood and is thought to transported across the blood-brain barrier (BBB) via brain capillary endothelial cells (BCECs) via a unique mechanism: namely, receptor-mediated endocytosis of Tf-iron from the blood followed by reduction of iron inside BCEC endosomes, followed by retro-endocytosis of apo-transferrin to the luminal surface [60, 63–65]. Ferrous iron is transferred from the endosome into the cytosol by DMT1 (similar to the classical receptor-mediated endocytosis mechanism of Tf-iron uptake in peripheral tissues), transported to the abluminal side of the BCECs, then exported across the abluminal membrane by ferroportin in a process involving subsequent re-oxidation of the iron to Fe(III) on the extracellular face of the abluminal membrane by the ferroxidases, ceruloplasmin [66] and/or hephaestin [67]. The resulting low-M_r iron is then thought to be complexed by endogenous iron-binding ligands, such as ATP, ascorbate or citrate, which are released by the end-feet of vicinal astrocytes [63]. The iron is then thought to be imported by the end-feet of these astrocytes [60], prior to its redistribution and subsequent trafficking within the brain parenchyma.

While the mechanisms responsible for the uptake of non-Tf iron by astrocytes are not known with certainty [61], at least two major routes of import have been proposed (recently reviewed in Codazzi et al. [68] and Skjørringe et al. [63]). Historically, the first is via DMT1, which has been observed to be highly expressed in astrocytic end-feet in culture [69–73], as well as *in vivo* in some studies [74, 75]. DMT1 levels are acutely regulated by cell iron-status in primary astrocyte cultures [70]. Moreover, astrocytes can release ascorbate to promote the uptake of iron by DMT1 under standard culture conditions [53], with the release of ascorbate being enhanced under conditions of hyperglutamatergia [52]. Collectively, these findings support a role for DMT1 in iron uptake *in vitro*. However, the involvement of DMT1 in astrocytes *in vivo*, at least under physiological conditions, is less clear (see references in Skjørringe et al. [63]). Notably, under conditions in which intracellular ascorbate is depleted (mimicking chronic oxidative stress), cultured astrocytes demonstrate an apparent preference for the uptake of Fe(III) [53], although the molecular pathway for the putative import of this Fe(III) has yet to be characterized.

Another proposed route for astrocytic ferrous iron uptake *in vivo* involves transient receptor potential canonical (TRPC) channels, based on studies conducted with quiescent hippocampal astrocytes [76]. Interestingly, astrocytes that have been activated by proinflammatory cytokines (i.e., IL-1β+TNFα) demonstrate a potentiation of non-Tf iron uptake by the *de novo* expression of the cell-surface DMT1-1A isoform [76], which is the same isoform expressed on the apical membrane of enterocytes, and are required for dietary uptake of low-M_r iron [77]. Accordingly, the *de novo* expression of this isoform of DMT1 in activated astrocytes was proposed to account for their increased capability to import Fe(II), but not Fe(III) [10, 78, 79]. Thus, the discrepancies on the importance of DMT1 in astrocytic iron uptake might be ascribed to variation in culture conditions that differentially activate astrocytes [80]. However, and perhaps more importantly, these findings suggest that inflammatory mediators can have profound effects on glia-regulated iron trafficking in the brain, which may be crucial for understanding how iron dysregulation occurs and progresses in AD.

In summary, astrocytes are critical in processing and re-distributing iron upon its entry into the brain across the BBB. Under physiological conditions, astrocytes can import non-Tf iron, which probably occurs *via* TRCPs, but with an increasing component of DMT1A-mediated iron uptake under conditions of neuro-inflammation, which may be relevant to the role of iron in AD.

IRON IS INCREASED IN THE AD BRAIN: A CONVERGENT PATHOLOGY

Iron is important for maintaining the high energy and metabolic requirements of neuronal tissues in the brain through its involvement in myelin synthesis and neurotransmitter synthesis (e.g., dopamine, serotonin, GABA) and for metabolism [3]. Increased

iron content in affected areas of the brain is observed in a growing number of neurodegenerative disorders including Parkinson's disease, Huntington's disease, and AD [3, 81, 82]. In the case of AD, elevated brain iron was first demonstrated in 1953 [83], and remains a widely and consistently reported finding [83–92]. Importantly, in AD, high Aβ-burden (identified by PET) predicts cognitive decline [93], but the large variability between individuals in the rate of this cognitive decline points to the contribution of other pathologies that synergistically combine with Aβ to accelerate clinical deterioration [94]. The accumulation of brain iron, which is a pathological feature of AD [25], has the potential to promote neurodegeneration through oxidative damage to sensitive subcellular compartments (discussed further below). Indeed, we have shown that elevated CSF ferritin (a biomarker of brain iron burden) predicts poorer cognition and increases the risk of developing AD [95, 96]. This notion of "convergent pathologies" in AD suggests that increased brain iron might combine with increased Aβ, or tau pathology, to increase the rate of disease progression. In support of this model, we recently employed quantitative susceptibility mapping to show that increased iron loading in the hippocampus is a strong predictor of Aβ-related cognitive decline [94].

Iron deposition within the brain parenchyma, particularly in vulnerable neuronal populations (e.g., within the hippocampus and cortex), but also in astrocytes, oligodendrocytes, and microglia, potentiates oxidative stress *via* the Fenton- and Haber-Weiss reactions (see above), as well as by increasing lipid peroxidative stress [19, 20, 23, 97–99]. The iron-dependent increase in general oxidative stress, particularly of membrane lipids in neurons and glial cells, is increasingly becoming accepted as a keystone contributor to the elevated signs of oxidative stress in the AD brain [100].

Iron enhances Aβ production and oligomerization

As discussed further below, elevated iron in the AD brain contributes to classical features of AD pathology, including Aβ dysfunction and plaque formation [101–106], tau hyperphosphorylation and neurofibrillary tangles [92, 107–111], as well as neuronal cell death [112, 113]. An increase in neuronal iron in AD is known to augment Aβ production by several mechanisms, including increasing AβPP expression and its subsequent amyloidogenic processing [106]. First, iron increases the translation of AβPP by virtue of an IRE in the 5′-UTR of its encoding mRNA [114]. This mechanism is essentially the same mechanism by which iron increases the expression of ferritin and ferroportin, both of which possess IREs in the 5′-UTR of their mRNA (discussed above). Thus, as with ferritin and ferroportin, the translation of AβPP, which is repressed by IRPs under low iron conditions, will be de-repressed under high cellular iron conditions (such as in AD), leading to increased translation of the transcript. Intriguingly, whereas the IREs in *ferritin* and *ferroportin* mRNAs can bind either IRP1 or IRP2, which is typical of all classical IREs [33], it has been recently shown by Jack Rogers' group that only IRP1 binds and regulates the IRE in the *AβPP* 5′-UTR [115]. Importantly, the selective regulation of the *AβPP* mRNA by IRP1 indicates that both AβPP and IRP1, the latter of which is deactivated as an IRE-binding protein by the acquisition of an 4Fe–4S ISC, may be regulated by the cytosolic (CIA) and mitochondrial ISC biogenesis pathway (for a recent review of the CIA and mitochondrial ISC pathways, see Paul and Lill [116] and Rouault and Maio [117]). The connection between AβPP regulation and ISC biogenesis is worthy of further investigation, particularly as other neurodegenerative diseases, such as Parkinson's disease and Friedreich's ataxia exhibit dysfunction in iron homeostasis that is coupled with mitochondrial dysfunction and aberrant ISC metabolism (for reviews, see [27, 118, 119]).

In contrast to AβPP, although the ferritin IREs can be bound by either IRP1 or IRP2 *in vitro* [33], in neural cells there is a preference for the binding of IRP2 to the IRE in the 5′-UTR of the *FTH1* mRNA [120]. This may be of relevance to the mechanism of neuronal iron dyshomeostasis and loading in AD, as IRP2, which is selective for ferritin IREs, has been observed to be dysregulated in AD [121]. Indeed, George Perry's group observed that while IRP1 is present at similar levels in both AD and control brain tissue, IRP2 shows marked differences in expression and localization, being associated with intraneuronal lesions, including neurofibrillary tangles, senile plaque neurites, and neuropil threads [121]. These findings suggest that an increase in IRP2 may contribute to the suppression of ferritin and ferroportin translation within neurons, leading to increased pools of redox-active iron through impaired storage and efflux, respectively. In support of this mechanism, the stabilization of IRP2 in neural stem progenitor cells by the genetic inactivation of the E3 ligase subunit, F-box/LRR-repeat protein 5 (FBXL5), which targets

IRP2 for proteasomal degradation, leads to the accumulation of ferrous and ferric iron, as well as the increased production of ROS [122].

In addition to promoting AβPP translation, high iron levels can increase amyloidogenic processing of AβPP, which occurs by the action of ferritin light chain binding to presenilin enhancer 2 (PEN-2), a γ-secretase component, and increasing γ-secretase activity [101]. Chronic iron loading increases amyloidogenic processing of AβPP leading, accelerating Aβ production and neurodegeneration in a mouse model of AD [123]. Importantly, Aβ accumulates in senile plaques, and engages in a positive feedback loop with oxidative stress that increases Aβ generation and oligomerization [124]. Additionally, Aβ is capable of binding transition metals (e.g., copper, zinc and iron), via three His (positions 6, 13, and 14) and 1 Tyr (position 10) residues that are located in the hydrophilic N-terminal region of the peptide [125, 126]. Interestingly, the redox potential of iron is significantly attenuated by Aβ, which may suggest a neuroprotective and chelating role for Aβ in AD pathogenesis that becomes toxic under certain conditions [9]. This feature of Aβ-iron interactions may, at least in part, explain the enrichment of iron in AD plaques that is observed in humans [127] and mouse models [128]. Interestingly, the metal-dependent generation of ROS by Aβ may be a good target for therapeutics. For example, chelation therapy using deferoxamine, a strong, but poorly BBB-permeant Fe(III) chelator, has shown improvement in several key indices in mouse models of AD (provided intranasally) [110, 129, 130], and has demonstrated clinical improvement in AD patients (provided intramuscularly, five days/week over two years) [131]. Critically, iron-stimulated aggregates of Aβ also demonstrate potentiated cytotoxicity *in vitro* [112, 132–135], suggesting that elevated iron and Aβ may synergistically combine to promote AD neuropathology. Moreover, the intranasal delivery of existing and novel iron-binding therapeutics may be a desirable route of administration, given the ability to bypass tight control by the BBB [136].

Iron enhances tau dysfunction and neurofibrillary tangles

Intriguingly, tau also binds iron [107, 108], which causes it to aggregate [109], possibly depositing *in vivo* as iron-rich tangles in AD brains [92]. In further support of a potentiating role for iron in tau dysfunction, iron-loading of cultured neurons increases tau phosphorylation [137–140], possibly by virtue of increased glycogen synthase kinase 3 beta (GSK3β) and/or cyclin-dependent kinase 5 (CDK5) activity (which could lead to increased tau phosphorylation), or loss of activity of the major tau phosphatase, protein phosphatase 2 (PP2A), which can occur under conditions of increased oxidative stress [141]. Iron-induced oxidative stress may also have a role in the tau hyperphosphorylation and polymerization. For instance, the oxidation of lipids, which is found to be elevated in AD brains, can facilitate tau polymerization, and may further drive oxidative stress and the formation of the tau fibrillar pathology in AD [142].

Consistent with the importance of iron in tau dysfunction, intranasal deferoxamine decreased the activity of GSK3β (a major tau kinase) in the AβPP/PS1 mouse model of AD, correlating with rescue of reference and working memory, and led to decreases in oxidative stress [130]. Importantly, total tau levels are decreased in AD cortex [143–146], and we recently demonstrated that loss of tau expression causes iron- and age-dependent cognitive loss and cortical atrophy in mice [147]. Tau is required for the correct trafficking of AβPP to the neuronal membrane [147], where it binds and stabilizes ferroportin in the cell membrane and facilitates iron efflux from neurons [90, 148], which is neuroprotective [149]. Consequently, reduced tau or AβPP levels could lead to iron retention in neurons that is observed in AD. Collectively, such findings suggest that elevated iron promotes pathological alterations in tau behavior in AD. Thus, while AβPP and tau play crucial roles in maintaining iron efflux from neurons [90, 150], chronic iron loading potentiates amyloidogenic processing of AβPP, the toxicity of Aβ aggregates, and tau dysfunction, which further increase iron-mediated lesions and neuropathologies.

Iron enhances neuronal cell death: Apoptosis

Iron-induced oxidative stress has been shown to initiate several apoptotic signaling pathways in neurons [151], and cause oxidative damage to key proteins such as Ca^{2+}-ATPase [152–155], glutamate transporter [19, 156, 157], ApoE [158, 159], Na^{+}/K^{+}-ATPase [152, 155, 160, 161], as well as the NMDA receptor [162–164], and lipids, such as cholesterol [165–167], ceramides [168, 169], polyunsaturated fatty acids (PUFAs) [98, 170–172], and sphingomyelin [173, 174]. There is extensive evidence that oxidative damage to proteins and lipids

by iron can cause synaptic dysfunction and neuronal cell death [175], both of which are critical features of AD.

Notably, the type of cell death that occurs in affected areas of the AD brain is still contentious, despite the demonstration that DNA fragmentation and upregulation of pro-apoptotic proteins has been frequently observed (for a review, see [113]). As such, it remains unclear to what extent apoptosis or emerging types of regulated necrosis (e.g., ferroptosis; see below) are responsible for bulk neuronal loss in AD [94]. Human AD brains show a 30- to 50-fold increase of DNA fragmentation in neurons and glial cells, compared to age-matched controls [113], and AD is characterized by dysfunctional DNA repair systems, leading especially to the accumulation of double strand breaks [5]. However, at least on the basis of DNA fragmentation, nuclear alterations suggestive of apoptosis have been reported to be rare in degenerating cells in AD (including neurons, microglia, and oligodendrocytes), except for those that are associated with Aβ deposits and neurofibrillary tangles of tau [176]. These observations suggest that apoptosis may contribute to cell death resulting in AD, even though other studies suggest that degenerating nuclei adjacent to Aβ deposits may not be apoptotic [177]. As an additional consideration, although DNA fragmentation is a classical feature of apoptosis (e.g., as measured by the TUNEL assay), this process can also occur in various models of regulated necrosis, particularly those that are associated with lipid peroxidative damage and glutathione depletion [178]. Such findings suggest that, in addition to apoptosis, other modes of cell death may also be relevant to neurodegeneration in AD.

The emerging role of ferroptosis in neurodegeneration

Although the evidence overwhelmingly suggests that elevations of redox-active iron in vulnerable brain regions in AD (e.g., hippocampus) clearly contribute to neurodegenerative processes and neuronal loss (as discussed above), the precise molecular pathways of cell death, and how iron is involved, remain unclear. Intriguingly, ferroptosis is an emerging pathway of iron-dependent programmed cell death, which is being currently investigated as a possible patho-mechanism in AD [179, 180]. This recently described mode of regulated necrosis was officially discovered and named in 2012 [181], and is distinct from all other known cell death modalities [20, 179, 181]. Essentially, ferroptosis is an iron- and lipid-peroxidation-dependent pathway of regulated necrotic cell death [182], which exhibits a unique dependence on RAS-RAF signaling, concurring with the original identification of the pathway in RAS-active cancer cells [181]. While an in-depth discussion of ferroptosis is outside the scope of this review, and the field is advancing rapidly, readers are referred to the following recent reviews [179, 183, 184].

Distinct from other types of cell death, ferroptosis nonetheless exhibits some key dependencies, and/or cross-talk, with other pathways (e.g., autophagy [185, 186] and apoptosis [187, 188]). In a pharmacologic setting, ferroptosis can be initiated by structurally diverse small molecules [179, 184], such as erastin (which inhibits cystine import by system Xc-), sulfasalazine (also inhibits system Xc-), and RSL3 (which inhibits the LOOH-detoxifying selenoenzyme, glutathione peroxidase 4 [GPX4]). Conversely, ferroptosis can be inhibited by: 1) lipophilic antioxidants, such as vitamin E, Trolox and; 2) redox-inactive iron chelators, such as deferoxamine; and 3) the small-molecule aromatic amine inhibitors, ferrostatin-1 and liproxstatin-1 [179, 184], as well as by lipid-soluble diarylamine radical-trapping antioxidants [189] and 1,8-tetrahydronaphthyridinols [190].

Loss of the activity of GPX4, a key glutathione-dependent enzyme specifically involved in protecting cells against ferroptosis, promotes the accumulation of membrane-associated LOOH [179, 184]. These LOOH can form spontaneously in the presence of existing lipid-reactive radicals and dioxygen (of ten termed "autoxidation"), which is driven by the presence of catalytic concentrations of labile iron, or can be enzymatically produced by the action of the non-heme iron lipoxygenases, ALOX12 or ALOX15, which drive ferroptosis through peroxidation of specific phospholipid-associated PUFAs at the bis-allylic position [191].

Recently, Kagan et al. [192] discovered that ferroptosis involves a highly organized oxygenation center, in which oxidation within ER-associated membrane compartments specifically targets phosphatidylethanolamines (PEs), and moreover, is specific towards two fatty acyls derived from arachidonic acid (AA) and adrenic acid (AdA). Moreover, suppression of AA or AdA esterification into PEs by genetic, or the pharmacological inhibition of acyl-CoA synthase 4 (ACSL4), inhibits ferroptosis [192, 193]. The implicated lipoxygenases (i.e., ALOX12/15) can generate doubly and triply-oxygenated (15-hydroperoxy)-diacylated PE species

that then act as specific ferroptotic signals, whereas tocopherols and tocotrienols (forms of "vitamin E") are able to suppress this activity [192]. Indeed, vitamin E is an important endogenous and physiological regulator of ferroptosis, which inhibits the process by directly inhibiting lipoxygenases [192] and/or acting as a membrane-soluble radical-trapping antioxidant [189]. Such considerations are of considerable relevance to AD, as some evidence suggests that vitamin E may be able to delay functional decline in patients with mild to moderate AD [194], as well as showing benefit in animal models of AD [195], although the role of vitamin E in protecting against AD is still under debate [196, 197].

In the context of cancer, ferroptosis may act as an endogenous tumor-suppressive mechanism downstream of p53 that acts, at least in part, by intracellular glutathione depletion (i.e., by decreasing expression of the system Xc- subunit, SLC7A11, that imports cystine that is required for glutathione biosynthesis) [198], and/or by increasing polyamine oxidation [199a] (i.e., by increasing expression of the polyamine N^1-acetyltransferase, SAT1), followed by enhancement of lipid peroxidation. Consistent with these findings, a very recent study has shown that cellular iron depletion markedly suppresses expression of polyamine oxidase (PAOX) [199b], an enzyme that metabolically cooperates with SAT1. Suppression of PAOX would be predicted to decrease polyamine oxidation and suppress ferroptosis-associated lipid peroxidation. From the point of view of the CNS, ferroptosis has recently been implicated in the pathological cell death of brain tissues exposed to pathological levels of glutamate, as well as kidney and heart tissues subjected to ischemia–reperfusion injury [181, 183]. It is, therefore, of great interest to understand how this novel regulated cell death pathway is specifically regulated in the brain, particularly as emerging evidence suggest that the pathophysiology of a range of neurodegenerative diseases may be associated with excessive ferroptosis [99, 200], including AD [179, 180], Parkinson's disease [201], Huntington's disease [179], and ischemic stroke [202].

Recent animal studies, in which *Gpx4* was conditionally inactivated in neurons, suggest that ferroptosis can be involved in the degeneration of spinal motor neurons and midbrain neurons [203], as well as neurons in forebrain regions, including cerebral cortex and hippocampus that are severely afflicted in AD patients [180]. Indeed, the "Gpx4BIKO" mouse model, in which Gpx4 has been conditionally inactivated in forebrain neurons for 12 weeks (following tamoxifen treatment to trigger gene inactivation), exhibited significant deficits in spatial learning and memory function. Subsequent examinations of the cognitively impaired Gpx4BIKO mice revealed profound hippocampal neurodegeneration [180]. This neurodegeneration was accompanied by markers of ferroptosis, such as elevated lipid peroxidation, ERK activation, and elevated neuroinflammation [180]. Notably, when Gpx4BIKO mice were fed a diet deficient in vitamin E, the rate of hippocampal neurodegeneration and behavioral dysfunction were augmented, providing support for an important role for vitamin E in protecting neurons against ferroptosis. Furthermore, neurodegeneration in these mice could be inhibited by liproxstatin-1 (administered i.p.) [180]. These results strongly suggest that forebrain neurons are susceptible to ferroptosis, particularly in the context of loss of GPX4 activity, further suggesting that ferroptosis may be an important neurodegenerative mechanism in AD.

In AD, the ferroptosis pathway may assist with understanding how iron potentiates the neurotoxicity of other key pathological hallmarks of the disease, such as those associated with Aβ and tau. It is tempting to speculate that the apparent co-dependence of AD pathology on elevated iron and Aβ [94], is that Aβ and tau dysfunction may potentiate the sensitivity of vulnerable neurons to ferroptosis, which could then activate under conditions of elevated iron and/or decreased glutathione and/or GPX4 activity. In support of this hypothesis, a recent study suggests that Aβ (specifically $A\beta_{42}$) increases RAS-ERK signaling and GSK3β activation, which the authors showed led to phosphorylation of AβPP at Thr668 (potentiating cleavage by γ-secretase) and tau [204]. Furthermore, the authors showed that RAS is hyperactivated in human postmortem AD samples compared to healthy controls [204]. As ferroptosis shows a dependence on RAS activation [181, 205], it may be the case that elevated brain iron "converges" with other key AD pathologies (e.g., those associated with Aβ and tau) that prime neurons for ferroptosis. Consistent with the convergent pathologies hypothesis, the ferroptotic "scales" in AD may be tipped in favor of neurodegeneration and overt neuronal loss in the context of elevated redox-active iron, depletion of cellular antioxidant reserves (e.g., glutathione and vitamin E), loss of GPX4 activity, and/or neuroinflammation that promotes further iron accumulation and oxidative stress.

CONCLUSIONS

Iron is a transition metal that is vital for life and is abundant in the brain, where it plays many vital metabolic roles. Iron is also "Janus-faced", as its enormous biological utility in being able to readily redox cycle between Fe(II) and Fe(III) states also endows it with the potential to "rust" brain tissue by producing ROS that lead to neurodegeneration and facilitate cell death. Iron contributes to AD pathology at numerous levels, and presently represents a promising and tractable target with untapped disease-modifying potential. Recent evidence points toward a possible role in AD for ferroptosis, a unique mode of programmed cell death that is dependent on redox-active iron and lipid-peroxidative stress and can occur in brain neurons. While this cell death pathway is only beginning to be explored in neurodegenerative diseases, it appears to have important and wide-ranging therapeutic implications for AD, particularly since ferroptosis can be readily prevented by iron chelators and endogenous and synthetic inhibitors of lipid peroxidation [205, 206]. Indeed, drugs specifically targeting components of the ferroptosis pathway (including iron) may show great promise in the treatment of AD and are worthy of further investigation.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9944).

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Brain Aging and Late-Onset Alzheimer's Disease: A Matter of Increased Amyloid or Reduced Energy?

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Abstract. Alzheimer's disease (AD) represents the most common form of dementia in old age subjects, and despite decades of studies, the underlying etiopathogenetic mechanisms remain unsolved. The definition of AD has changed over the past years, offering an ever more detailed definition of pre-morbid and pre-clinical status, but without a similar strong emphasis on the role of aging as the main risk factor. In fact, while early-onset AD is a clear consequence of gene mutations, late-onset AD is more likely due to a gradual accumulation of age-related damages. The pathogenetic amyloid cascade hypothesis has been recently questioned due to multiple clinical failures. Furthermore, several studies reported that cognitively normal elderly have a high amyloid deposition in the brain comparable to the levels observed in old age subjects with AD. This suggests that amyloid accumulation enters into the normal process of aging and what really triggers neuronal death and clinical manifestation in late-onset AD still needs further explanation. In this context, 'normal brain aging' and AD might represent a different pathway of successful or failed capability to adapt brain structures and cerebral functions. Cellular senescence and age-related changes affecting the brain may be considered as biologic manifestations of increasing entropy. Bioenergetic deficits due to mitochondrial dysfunction may lead to progressive neuronal death and clinical expression of dementia. So, increased amyloid in the brain of old age subjects may represent the downstream event expression of a biological system that is cooling down because of its exhaustion and not the core causative factor of late-onset dementia.

Keywords: Aging, Alzheimer's disease, amyloid, energy, entropy, mitochondria, old age

A BRIEF HISTORICAL VIEW

Over 110 years have passed since Alois Alzheimer first described the pathology and symptoms of a young subject with dementia whose brain contained characteristic and histopathologic features called 'neuritic plaques' and 'neurofibrillary tangles' [1]. In the same year, Oskar Fischer also detected neuritic plaques in brains of old age subjects suffering from dementia [2]. Upon these observations, the scientific community considered the first situation as a disease, named Alzheimer's disease (AD), and the second one as a consequence of aging, and thus defined senile dementia [3]. So, at that time, old age subjects with dementia were not diagnosed with AD, even though their brains frequently contained neuritic plaques and neurofibrillary tangles, as confirmed in postmortem studies. Upon such classification, in the following decades, AD was considered a rather uncommon entity of pre-senile dementia while senile dementia became progressively prevalent as life expectancy increased worldwide. In 1976, Katzman broke again what had become a certainty and with an editorial stated, "Alzheimer disease and senile dementia are a single process and should, therefore, be considered a single disease" [4, 5]. Since then, the definition

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changed and included all subjects, independently of age, with cognitive impairment as well as brain histopathological feature of AD.

After that, accumulation of epidemiologic data allowed researchers to identify two distinct AD populations: those with clearly recognizable autosomal dominant inheritance and those without evident genetic influence. The former typically present at younger ages and were thus defined as early-onset AD. The late onset group instead tends to manifest either sporadic or pseudo-sporadic epidemiology, considering that they are more likely to have AD-affected relatives.

Persons with autosomal dominant AD usually show clinical signs in their fourth or fifth decade of life. In this context, mutations in three 'deterministic' autosomal dominant genes have been identified. These genes include the amyloid precursor protein gene on chromosome 21, the presenilin 1 gene on chromosome 14, and the presenilin 2 gene on chromosome 1. Mutations in each gene increase production of the amyloid-β (Aβ) derivatives from the cleavage of amyloid-β protein precursor (AβPP) [6].

On the contrary, late-onset or pseudo-sporadic AD is not associated with deterministic gene mutations, but often genetically influenced. The most established genetic risk factor is the allele ε4 of the apolipoprotein E (*APOE*) gene on chromosome 19 [7], that is associated with the most common late-onset familial and with sporadic forms of AD. Although the mechanism by which APOE ε4 participates in pathogenesis is still under debate, the protein encoded by this gene is immunoreactive in plaques and neurofibrillary tangles that define the phenotype. The question of whether pathogenesis of 'early' and 'late' onset cases is similar enough to qualify them as a single disease was previously raised although not conclusively settled. Undoubtedly, they have many common traits, but they also exhibit numerous differences, as reported in Table 1.

However, the fact that they display a similar pathological process, which is the main diagnostic criterion for AD, led to the conclusion that they are technically variants of the same disease. Considering that the onset of cognitive deficits generally occurs within the 6th decade of life and severity increases along with time, advancing age represents the major known risk factor for AD. Interestingly, most people now diagnosed with dementia are old and would not have been diagnosed with AD as originally conceived. Accordingly, younger patients that qualify for a diagnosis of AD under both original and current AD constructs now represent an exceptionally small percentage of the diagnosed population.

In 1985, Stewart Shapiro and collaborators wrote a review entitled "Alzheimer's disease: an emerging affliction of the aging population" stating that "the number of people who will have Alzheimer's disease will double by the year 2030 because of the rising elderly population". Again, they concluded, "Currently, there is no agreement relative to the etiology of Alzheimer's disease, no effective cure, and no effective symptomatic therapy."

THE AMYLOID HYPOTHESIS AND THE AGING BRAIN

Because of the progressive aging of population thanks to significant increase in life expectancy worldwide, current projections on incidence and prevalence of dementia look worse and scary [8]. Nowadays, AD represents the sixth leading cause of death in the USA, with five million subjects with AD, that could triplicate in three decades. The reason why the aging brain is particularly and extremely susceptible to dementia and what features can distinguish age-associated brain changes from those typical of AD is still unclear. Despite a long-lasting research in this area, the underlying mechanisms that trigger such a neuropathology remain unresolved.

The most supported and established pathogenetic hypothesis of AD in recent years is the so-called "amyloid hypothesis". It postulates that high levels Aβ, in a variety of forms, but mainly as $A\beta_{42}$, triggers a cascade of events producing the pathological presentations of Aβ plaques, tau tangles, synapse loss, and neurodegeneration, which induces cognitive impairment. In detail, Aβ is a proteolytic degradation product of the larger amyloid-β protein precursor (AβPP), that can easily aggregate. Proteolysis by α-secretase can occur 83 amino acids from the AβPP intracellular carboxyl-terminal [9–12]. Alternatively, proteolysis by β-secretase (BACE1) cuts 99 amino acids upstream of the AβPP carboxyl end. An enzyme complex, the γ-secretase, further processes the remaining carboxyl end of α-secretase (C-terminal fragment α; CTFα) or β-secretase (C-terminal fragment β; CTFβ) digested AβPP. In AβPP, mutations around the γ-secretase cleavage site cause a change in amino acids adjacent to the BACE1 cleavage site. PSEN-1 gene mutations (which give rise to proteins called presenilins) predominantly alter the amino acids in their nine transmembrane

Table 1
General characteristics of early and late-onset Alzheimer's disease

	Early-Onset Alzheimer's Disease	Late-Onset Alzheimer's Disease
Age at onset	Younger than 65 years	65 years and older
Progression	Faster	Slower
Neuropsychology	Poor writing tasks, executive functions, visuospatial functions, motor behaviors	Poor memory and language
Neuropathology	Greater and more diffuse distribution of senile plaque, neurofibrillary tangles, and neuronal loss	Senile plaque and neurofibrillary tangles, neuronal loss
Cerebrospinal fluid markers	Similar values in amyloid-β, total tau protein, and phosphorylated tau protein	
Genotype	Absence of ε4 alleles	Favored by 1 or 2 ε4 alleles
Structural and functional neuroimaging	Frontal/temporoparietal atrophy and decreased metabolism in temporoparietal cortex	Hippocampal atrophy; decreased metabolism in medial temporal lobe

Modified from van der Flier et al. [60].

domains. The common thread to all these mutations is an increased production of the less soluble and more toxic $A\beta_{42}$. Several studies using postmortem tissue from patients with AD have demonstrated the presence of soluble oligomeric Aβ species in AD brains [9–12]. Thus, oligomerization of Aβ has been proposed to be a key event in the pathogenesis of AD. Aβ is thought to go through a process of progressive aggregation from monomers to oligomers until plaque formation [13]. Recent evidence shows that soluble oligomeric species of Aβ have direct adverse effects, whereas fibrillar or monomeric Aβ seems to be less harmful *in vitro* [14–20] and in animal models [21–24]. Aβ oligomers are in fact responsible for synaptic dysfunction and for initiating processes leading to cell death and neurodegeneration. Indeed, studies using stable isotope labeled kinetic (SILK) techniques have recently better clarified that the main abnormality of Aβ in late-onset AD is a reduced clearance, in contrast with the autosomal dominant form of early-onset AD, where mutations in the AβPP or presenilin component of γ-secretase result in an overproduction of Aβ [25]. Amyloid depositions are part of the histopathological definition of AD, and thus much effort has been made on *in vivo* biomarkers of amyloid in contemporary AD research, as reflected in the NIA-AA proposed diagnostic guidelines for AD and its preclinical stages [26, 27]. They state that brain alteration in AD start years before clinical symptoms, causing neuronal functional damage and then clinical manifestation [27]. However, it has been suggested that once initiated, neurodegeneration in AD progresses independently of its amyloid-trigger, leading to the commonly expressed concern that the therapeutic window for anti-amyloid drugs is quite narrow, particularly when the amyloid cascade starts to accelerate and neurodegeneration become irreversible.

The recent failures of drugs targeting amyloid pathways have raised questions not only about this approach but also on the validity of the amyloid hypothesis itself. Moreover, studies of oldest-old individuals indicate that the occurrence of AD dementia is not a mandatory phenomenon of increasing chronological age. Approximately 20% to 30% of cognitively normal elderly have a similar amyloid deposition in the brain compared to the levels observed in AD dementia [28]. To further complicate the story, neuritic plaques also occur in cognitively healthy old age subjects. In old-age subjects with dementia, amyloid levels in cerebrospinal fluid (CSF) and amyloid cerebral load in PET-imaging do not correlate with cognitive decline [29]. Measurement of $A\beta_{1\text{-}42}$ in CSF shows reduced levels already in the preclinical phase of AD that remain low throughout the prodromal and dementia phases [30]. Similarly, amyloid imaging has confirmed that amyloid deposition begins before significant cognitive symptoms occur and Aβ burden in the brain remains approximately the same throughout the remainder of the disease [31]. Among old age subjects, there are patients with evident, sometimes severe, clinical expression of AD, but with low brain amyloid pathology while subjects with cognitive complaints, not severe enough to meet clinical criteria for dementia, have a brain amyloid load compatible with the diagnosis of AD [32–36]. Some of them will die without becoming demented [37].

These observations suggest that in many old age subjects brain can tolerate a high amyloid accumulation without cognitive dysfunctions and, vice versa, that in old age patients with dementia other events are required to cause neurodegeneration and cognitive impairment. Moreover, autopsy studies of patients in the AN1792 Aβ vaccination trial showed that cognitive decline continues despite the effective

removal of Aβ plaques [38]. Indeed, a recent study demonstrated that neuroradiological, biochemical, and neuropathological measures of neurodegeneration do not correlate with each other in a cohort of very old men. These measures also do not reflect the cognitive performances, suggesting that biomarkers of AD are less informative in the oldest-old [39]. Altogether, these data suggest that while amyloid can be considered as a hallmark of AD in younger subjects, its relationship to cell dysfunction and cognitive decline in the elderly is not so consequential. Rather, it seems that in the old age amyloid accumulation enters into the normal process of aging and what really triggers neuronal death and clinical manifestation has not evaluated in detail yet.

An unresolved conundrum is why Aβ and Aβ oligomers can be resident in the brain for many years without producing sufficient detectable cognitive dysfunction. Possibly, oligomers need to reach specific concentrations or be present in the brain for prolonged periods of time before neurotoxicity is triggered. The relationship between Aβ and cell death in the course of AD requires further clarification. The repeated failures of clinical trials with molecules acting on amyloid have been justified by the inclusion of subjects with too advanced brain pathology, unresponsive to any therapeutic intervention. But, on the other hand, these results provide additional data suggesting that amyloid might not be causal in late-onset AD pathophysiology [40–47]. Although the oldest olds represent the largest and fastest growing population with dementia, most studies on dementia are focused on a younger population, in which amyloid is probably the only or the main cause of the disease. This is probably not true in the oldest-old, where other aspects, more related to the aging process at the molecular and subcellular level, better define the pathway leading to dementia.

For these reasons, it is necessary to better understand the relationship between amyloid, brain integrity, and cognitive function in healthy old age subjects. The core question is: what amyloid-related changes in the aging brain represent AD-related pathology, and what, if any, such changes can be expected as part of the 'normal' aging process? With this perspective, normal aging and AD might represent a different pathway of successful or failed capability to adapt brain structures and cerebral functions to aging processes. Thus, understanding their similarities and differences might be the key to solve such an enigma.

In this context, amyloid in the elderly may represent only a marker of the aged brain, which accumulates along with time and then contributing to, but not causing by itself alone, neuronal dysfunction. Therefore, some other mechanisms must be evaluated and put under the microscope.

AGING AS THE MAIN RISK FACTOR FOR OLD-AGE DEMENTIA: THE ROLE OF ENERGY AND MITOCHONDRIA

In order to re-formulate hypotheses on the pathogenesis of old-age dementia, we should put aging at the center of the debate. Aging is the inevitable biological process that results in a progressive structural and functional decline, from the cellular level to the whole body, causing a reduced ability to adapt to environmental changes and stressors. 'Cellular senescence' is one of the main contributing factors to age-associated cerebral dysfunction [48] and represents the core feature of the so-called age-related changes (ARCs) producing an overall reduction in the brain volume and weight and enlargement of cerebral ventricles [49]. Somatic cells are not able to proliferate indefinitely, but they arrest irreversibly after a limited number of divisions leading to complex changes in cellular metabolism, gene expression, and epigenetic regulation [50]. Increasing evidence shows that senescent cells are detectable in mammalian brains along with aging, and may also be implicated in neurodegenerative disorders [51]. For example, in brains of subjects affected by AD, microglial cells show a significant increase of biomarkers of senescence [52], which precedes the tau pathology in neurons [53]. These observations suggest that microglia are subjected to ARCs, and the impairment of microglial neuroprotective function is likely to have detrimental consequences for neurons, such as the development of neurofibrillary pathology. ARCs can occur in two fundamental ways: by a purposeful program driven by genes or by random, accidental events, both affecting brain cells viability and vulnerability. Intrinsic ARCs are those resulting from the programmed neuronal decline or due to the accumulation of waste byproducts. Extrinsic ARCs are the result of stochastic damaging events that can reduce the effective functioning of the brain below its expected duration.

The effects of such changes can be seen as the biological manifestation of increasing entropy of the

system—defined as a measure of disorder according to the second law of thermodynamics. Entropy is the tendency for concentrated energy to disperse. The hindrance of entropy change is the relative strength of chemical bonds. The prevention of chemical bond breakage, among other structural changes, is essential for life. Through evolution, natural selection has favored energy states capable of maintaining fidelity in most molecules until reproductive maturation, after which there is no value for those energy states to be maintained indefinitely to keep alive a not reproductive organism. So, the aging process occurs because the decreased energy state alters structure and function of biomolecules leading to a progressive cellular damage and inactivity, until death.

Disruption of energy metabolism is commonly observed in senescent cells. In this context, mitochondria have a central role in the energy metabolism, representing the coal power plant. Most of the energy derived from the oxidation of nutritional substrates by the mitochondrial respiratory chain and transformed into ATP, the cellular energy currency. Aging is characterized by increased levels of mitochondrial DNA mutations, a declined function of the respiratory chain and abnormal mitochondrial elongation, likely due to increased expression of mitochondrial fusion proteins [54]. Overall, structural as well as functional abnormalities of mitochondria may lead to reduced energy level and to enhanced cellular damages which in turn leads cells to senescence or apoptosis. The decline of energy production causes an increased entropy, and biological aging represents the biomedical counterpart of the irreversible increasing entropy of any living system (cell, tissue, organ, body) where ARCs are the specific molecular components. Thus, along with aging, as entropy increases in the brain, the biological processes that normally maintain its structure and function start to decline, and altered misfolded proteins start to accumulate. Therefore we could hypothesize that the protein misfolding and aggregation we observed in aging brain, and then in late-onset dementia, is the final effect of a reduced energy production, due to exhausted mitochondria, and an increased entropy in the brain.

The impact of increasing entropy on the aging brain is highly visible for its unique complexity and shaped not only by the brain specialized neural functions, but also by the many ARCs, and opposing intrinsic and extrinsic homeostatic mechanisms. In this context, diet, lifestyle, and education may strongly modify the speed and the course of the process. However, when deterioration exceeds the capacities of these modulating factors, a progressive but irreversible functional decline appears.

One intriguing feature of the physiological aging the mammalian brain aging is the relatively slow rate of neuronal loss compared to the greater rate of decrease of cerebral myelinated nerve fibers [55]. However, when senescent neurons start to accumulate, the homeostatic equilibrium shifts from a gradual and linear decline to an accelerated degeneration. By recognizing sporadic late-onset AD as a disorder linked to senescence, driven by an increasing entropy and due to ARCs, several approaches to the understanding of the etiology and proposal of specific treatment could be scientifically re-evaluated.

The concept of late-onset AD as a consequence of increasing entropy—with an accelerated, catastrophic decline when homeostatic mechanisms fail—suggests that strategies designed to modify the course should precede the shift from gradual decline with normal aging to rapid tissue loss with AD. Thus, it seems important to reconsider late-onset AD as a complex condition with a prolonged trajectory of changes in the brain, characterized by progressively reduced metabolism and impaired bioenergetics. These changes start many years before the clinical onset, what supports and, in the meantime reflects, the incapacity of a biological system to maintain the molecular order that guarantees life thanks to a constantly high energetic support.

In this view, considering the fundamental role of mitochondria in cellular bioenergetics, the decline in mitochondrial function represents probably the pivotal factor. The 'mitochondrial cascade hypothesis' places the mitochondrial dysfunction as the leading factor in the pathological cascade of late-onset AD, underlying the individual genetic background able to regulate since birth its mitochondrial function and sustainability. When the mitochondrial function declines and falls below a critical threshold, AD-typical dysfunction may ensue at the cellular level, including Aβ production, tau phosphorylation, synaptic degeneration, and oxidative stress [56–58]. In fact, perturbations in mitochondrial function have long been observed in samples derived from clinically confirmed AD, including altered mitochondrial morphology, compromised enzyme complexes in the tricarboxylic acid cycle, and reduced cytochrome c oxidase activity protein (reviewed in [57]). Moreover, Aβ accumulates within mitochondria and interacts with mitochondrial proteins (reviewed in [59]). All

these processes create a vicious cycle in which excessive Aβ accumulation and sustained mitochondrial dysfunction synergize to activate a cascade of neurodegenerative pathways [59]. This unique trajectory enables a bioenergetic-centric strategy that targets disease-stage specific profile of brain metabolism for disease prevention and treatment: it depends on modifying as many ARCs as possible to delay and slow the increasing disorder due to entropy and avoid loss of brain function and increased neural vulnerability as long as possible.

In this perspective, the progressive reduction of capacity in producing, storing, and maintaining a high energy level, which is the main strategic role of mitochondria in eukaryotic cells, reflects the increased entropy that progressively leads the organism from function to dysfunction and then to death, the expression of the maximal entropic status. Reconsidering late-onset AD as a matter of energy rather than as a matter of amyloid could open new perspectives regarding pathogenesis and, overall, regarding prevention and therapy. Some strategies for delaying ARCs already have been identified such as avoiding vascular risks or limiting oxidative stress production. Many others may represent attractive targets against neurodegeneration.

In conclusion, the role of aging as a progressive status of energy decline can represent the key to recollect many theories around the main phenomenon that characterizes life: the fatal attraction toward its end.

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Metabolic Dysfunction in Alzheimer's Disease: From Basic Neurobiology to Clinical Approaches

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Abstract. Clinical trials have extensively failed to find effective treatments for Alzheimer's disease (AD) so far. Even after decades of AD research, there are still limited options for treating dementia. Mounting evidence has indicated that AD patients develop central and peripheral metabolic dysfunction, and the underpinnings of such events have recently begun to emerge. Basic and preclinical studies have unveiled key pathophysiological mechanisms that include aberrant brain stress signaling, inflammation, and impaired insulin sensitivity. These findings are in accordance with clinical and neuropathological data suggesting that AD patients undergo central and peripheral metabolic deregulation. Here, we review recent basic and clinical findings indicating that metabolic defects are central to AD pathophysiology. We further propose a view for future therapeutics that incorporates metabolic defects as a core feature of AD pathogenesis. This approach could improve disease understanding and therapy development through drug repurposing and/or identification of novel metabolic targets.

Keywords: Alzheimer's disease, hormones, memory, metabolism, therapy

INTRODUCTION

The segment of population comprising people aged 60 and older is the fastest growing worldwide, and it is expected to more than double in the next 35 years [1]. However, living long does not necessarily mean living well, as age-associated diseases emerge and, for instance, the number of deaths caused by Alzheimer's disease (AD) has considerably risen [2], despite some evidence for stable or declining incidence of dementia [3]. In fact, it is estimated that 47 million people live with dementia worldwide, causing huge economic and social hurdles.

AD is the most common cause of dementia, affecting around 60–70% of demented patients [4–6]. Although it primarily affects cognition, other debilitating non-cognitive symptoms may emerge including psychosis, mood alterations, impaired

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wake-sleep pattern, and appetite changes [7, 8], making AD an expensive and painful disease not only for patients, but also for caregivers and society. Recent works further suggest that central and peripheral metabolic disturbances, including insulin resistance and impaired glucose uptake, play key roles on AD risk and onset [9–11].

During the past decades, extensive basic and clinical research has expanded our knowledge on the cellular and molecular aspects of AD, contributing to the development of novel therapeutic approaches and tools for early diagnosis. However, even therapies initially considered extremely promising have been found somewhat disappointing in clinical trials [12, 13].

Recent results from Sevigny and co-workers suggest that the anti-aggregated amyloid-β (Aβ) antibody aducanumab reduces Aβ burden in a dose-dependent manner and causes slight improvements in memory scores in patients with prodromal or mild AD [14]. Conversely, several anti-amyloid strategies have failed to offer benefits to patients, and some clinical trials have even been halted due to safety concerns. For instance, the anti-Aβ antibody solanezumab failed in a large clinical trial tracking more than 2,100 people diagnosed with mild dementia due to AD for 18 months [15]. This suggests that current AD drug discovery pipeline might not be precisely addressing disease mechanisms. Still, additional candidate drugs with a wide range of mechanisms, including BACE and γ-secretase inhibitors, blockers of tau aggregation, and active and passive immunization strategies are currently in various stages of development and clinical trials, meaning there is hope that novel approaches will emerge shortly.

Mounting epidemiological studies and experimental evidence have supported a link between metabolic disorders and AD [10, 16–23]. A number of pilot clinical trials have recently indicated that drugs that modulate metabolism, including insulin and glucagon-like peptide-1 (GLP-1) analogs, may confer improvements in AD symptoms [24–26]. Hence, drugs targeting metabolic defects may have important therapeutic implications in AD.

Obesity and type 2 diabetes mellitus (T2DM) have been regarded as core metabolic disorders related to AD, as they share demographic profiles, risk factors, and clinical/biochemical features. Notably, both obesity/T2DM and AD have been associated with chronic inflammation, oxidative, and endoplasmic reticulum (ER) stress, and reduced neuronal sensitivity to insulin. Such molecular alterations are accompanied by energy metabolism deregulation and impaired glucose uptake [10, 27–32].

Since the emergence of initial studies proposing abnormal brain glucose metabolism as an important player in AD [33, 34], metabolic impairments that are hallmarks in diabetes and obesity have been postulated as core events in dementia [30, 35, 36]. Indeed, PET-based measures have described abnormal glucose utilization in AD brains [26, 37] and evidence from *ex vivo* studies using AD tissues established that demented brains are less responsive to insulin/IGF-1 stimulation than controls [38]. Additional findings have demonstrated early Aβ deposition, decreased glucose metabolism, structural changes, and functional disruption at the same cortical midline brain regions vulnerable to AD changes [39–42]. This notion has been substantially reinforced after other molecular hints indicated that inflammation and defective insulin signaling are present in AD brains [10, 29–31].

Accumulating reports have further established that life habits, feeding behavior, and environmental factors throughout life could contribute to increase susceptibility to sporadic AD [10, 43, 44]. Obesity, for example, is associated with poorer cognition in non-demented subjects [45], and comprises a risk factor contributing to AD development [46]. A large-scale study that followed 10,136 participants for 36 years reported that participants that were overweight in midlife had three-fold increased risk to develop AD than those with normal weight [47]. Interestingly, this study found an association between obesity and AD even after corrected covariation for hyperlipidemia, hypertension, and diabetes, suggesting that body weight is an independent risk factor for AD. Another study conducted by the Cardiovascular Health Consortium also found positive associations between body mass index and AD, reporting that obesity in midlife was associated with a 40% increase in the risk for developing this form of dementia [48].

High adiposity has been associated with alterations in brain structure in late-life, such as brain atrophy and white matter lesions especially in brain regions involved in memory processing, such as the amygdala, hippocampus and frontal cortex [49]. Elevated fat consumption was also shown to increase levels of soluble and insoluble Aβ in the parietal-temporal cortex of aged transgenic AD mice as compared to wild-type animals, indicating that high adiposity could accelerate AD pathology [50]. Thus, controlling obesity and T2DM throughout midlife could

represent a modifiable risk factor not only for cardiovascular and metabolic disease but also for AD, and future studies are warranted to explore such interventional approaches.

Therefore, attempts to halt metabolic defects in early stages of AD, as well as strategies aimed at preventing metabolic deregulation, could be key to slow AD progression or even reduce its risk. Here, we review clinical and preclinical evidence supporting metabolic deregulation as a core derangement in AD, and discuss potential therapeutic implications of such findings. Finally, we offer a perspective for future therapeutic approaches that takes metabolic dysfunction into account in AD.

METABOLIC DEFECTS IN AD

Mitochondrial dysfunction

Mitochondria play pivotal roles in cell survival by regulating energy metabolism, reduction-oxidation potential, and apoptotic pathways [51]. They have recently been demonstrated to actively participate in neurotransmission by locally controlling ATP and metabolite levels at synapses [52–55]. Thus, it is conceivable to speculate that alteration of mitochondrial structure, localization, and function could affect neurotransmission and neuronal function, ultimately impinging on cognition.

Current evidence suggests that mitochondrial abnormalities and oxidative damage are early events in AD, and may precede pathological hallmarks [56–58]. AD brains present reduced expression and/or activity of key enzymes of mitochondrial oxidative metabolism, including α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and cytochrome oxidase [59–61]. Neurons from AD patients exhibit overall decrease in mitochondrial mass, aberrant mitochondrial DNA release to cytosol, and increased mitophagy [62–69]. Aβ-induced mitochondrial dysfunction further potentiates the opening of the mitochondrial permeability transition pore (PTP) induced by Ca^{2+} [70, 71], which contributes to the release of pro-apoptotic proteins, such as cytochrome c and apoptosis-inducing factor.

Neuronal oxidative stress, a consequence on mitochondrial dysfunction, has been extensively demonstrated in the brains of patients and in experimental models of AD [56–58, 67, 69, 72, 73]. Although physiological levels of reactive oxygen species are essential for brain function [74], neurons are especially sensitive to them, and prolonged oxidative stress may thus result in neurodegeneration [75, 76]. In line with this, blocking oxidative stress prevents AD-related neurotoxicity in AD models [62].

Mitochondria are highly dynamic organelles that undergo continual fusion and fission events, with impacts on mitochondrial biogenesis, morphology, trafficking, and degradation [77]. Mitochondrial fusion and fission events are imbalanced in AD [78–81], similar to obesity-related alterations [82], and experimental models have further revealed defective mitochondrial transport [31, 83] and increased fragmentation [62, 63, 78] in neurons undergoing AD-related neurotoxicity. These events likely contribute to the metabolic failure germane to AD.

Brain glucose metabolism

For more than three decades now, ^{18}F-fluorodeoxyglucose (FDG)-based PET has been used to demonstrate impaired glucose metabolism in AD, as compared to healthy subjects [84]. Such an approach has revealed that disease progression positively correlates with reduction of cerebral glucose metabolism with marked effects in areas notably affected by AD, including the posterior parietal lobe and portions of the temporal and occipital lobes [85]. Interestingly, APOE4 carriers, who are at higher risk of developing AD, present weaker FDG-PET signals decades before any clinical manifestation, suggesting that defects in brain metabolism may precede dementia onset [86]. Consistently, AD transgenic animal models also develop hypometabolic profiles in FDG–PET [87–89].

Still, the specific reasons that lead to reduced FDG signals in AD are unclear. An immediate explanation for altered FDG signals in AD brains could be decreased expression/function on glucose transporters. Indeed, impaired expression of GLUT1 and GLUT3 has been observed in AD brains [90, 91]. Reduced levels of glucose transporters are likely to contribute to synaptic dysfunction, tau phosphorylation [90, 92], and vascular pathology [93] in AD models. Conversely, increasing GLUT1 expression was shown to rescue Aβ-induced neurotoxicity [94, 95], further supporting its potential role in AD.

In line with the impaired glucose uptake in AD, glucose phosphorylation by hexokinase appears to be reduced during the course of the disease [96]. It is noteworthy that aerobic glycolysis was recently shown to be reduced during normal aging [97], and this could be exacerbated in AD. Mechanistic hints for such events may have found place in

that AD-associated soluble Aβ oligomers (AβOs) dampen hexokinase activity and reduce ATP levels in primary neurons in culture [65, 66]. Additionally, AβOs lead to transient inhibition of the metabolic sensor AMP-activated kinase (AMPK), which causes GLUT3 and GLUT4 removal from the neuronal surface [65]. Such metabolic responses could lead to compensatory longer-term increases in AMPK activity, thereby resulting in the aberrant AMPK overactivation described in the brains of AD patients and transgenic mouse models [98–101].

Thus, defective glucose uptake in AD brains could result from compromised metabolic routes, including perturbed exposure of GLUTs and impaired metabolic sensing by AMPK. Impaired neuron-astrocyte-vascular interactions and signaling could further exacerbate metabolic dysfunction, ultimately leading to the observed declines in FDG signals and brain function in AD patients.

Insulin resistance

An important player accounting for impaired glucose metabolism in AD could arise from defects in insulin signaling. Historically, the skeletal muscle, adipose tissue, and liver have been considered the main insulin-responsive tissues in control of peripheral metabolism. On the other hand, the brain was classically considered an insulin-insensitive organ until the initial observation that intracerebroventricular infusion of insulin reduces food intake and body weight in baboons [102].

In fact, insulin and insulin-like growth factor receptors are widely distributed throughout the encephalon [103]. The hippocampus and cortical formations present significant expression of theses receptors and are regions centrally involved in memory formation [104–106]. In accord, insulin was shown to be neuroprotective [31, 107–111], and to promote synapse plasticity [112, 113] and cognitive function in healthy subjects [108, 114–116]. Conversely, downregulation of brain insulin receptors was shown to promote tau phosphorylation [117], synaptic impairments, and memory loss [105, 118].

The sequence of events leading to brain insulin signaling dysfunction in AD is not completely understood, but resembles, in many aspects, the molecular steps described for T2DM in peripheral tissues. Hints into the mechanisms of neuronal insulin signaling dysfunction in AD came from experiment using primary hippocampal neurons showing that AβOs induced the removal of insulin receptors from the surface of neurons, an effect that was prevented by insulin itself or by insulin-sensitizing drugs [110]. Recently, tau deletion was shown to promote brain insulin resistance through aberrant PTEN activity, arguing for a role of tau loss-of-function in the deleterious effects in AD [119]. Further, *ex vivo* insulin stimulation in slices derived from human AD brains revealed an impairment of insulin signaling compared to tissue from age-matched controls [38]. Also, AD patients exhibited increased levels of serine phosphorylation in the insulin receptor substrate 1 (IRS-1 $pSer^{616}$ and IRS-1 $pSer^{636/639}$) that negatively correlated to memory scores [38]. This is in full accordance with early studies that established that expression and activity of brain insulin signaling components are reduced in AD [21, 31, 120–122]. Further, soluble Aβ-injected mice [109] and cynomolgus monkeys [31] present increased levels of IRS-1 $pSer^{636/639}$, in line with AD patient data. Thus, impaired brain insulin signaling could compromise survival and synaptic plasticity mechanisms, likely cooperating to memory defects in AD. Therefore, boosting the insulin signaling pathway in the brain may represent an important alternative strategy for AD treatment (Fig. 1).

Brain inflammation

Preclinical, clinical, and epidemiological evidence has indicated that inflammation is an important contributor to AD pathogenesis. Several studies have shown that markers of inflammation are increased in the brains, cerebrospinal fluid, and plasma of AD patients [123, 124]. These include TNF-α, IL-1β, IL-6 and other cytokines, as well as indicators of glial reactivity and infiltration of peripheral immune cells [125–128].

Although predicted in the original amyloid cascade in the early 1990s, only recently detailed insights on how brain inflammation may take place in AD came out. Microglia has been placed at the center of AD-linked inflammation and, while normal microglial activation is fundamental for Aβ clearance, chronic inflammation generates detrimental effects that promote AD pathology [125, 126, 129]. Current notions suggest that microglial function go awry, resulting in increased pro-inflammatory signaling, reduced Aβ clearance and aberrant synaptic pruning [125, 126, 130]. Microglia from AD mouse models present impaired phagocytosis capacity, degrade less Aβ, produce toxic signals, and exacerbate neuronal damage in AD models [126, 131, 132].

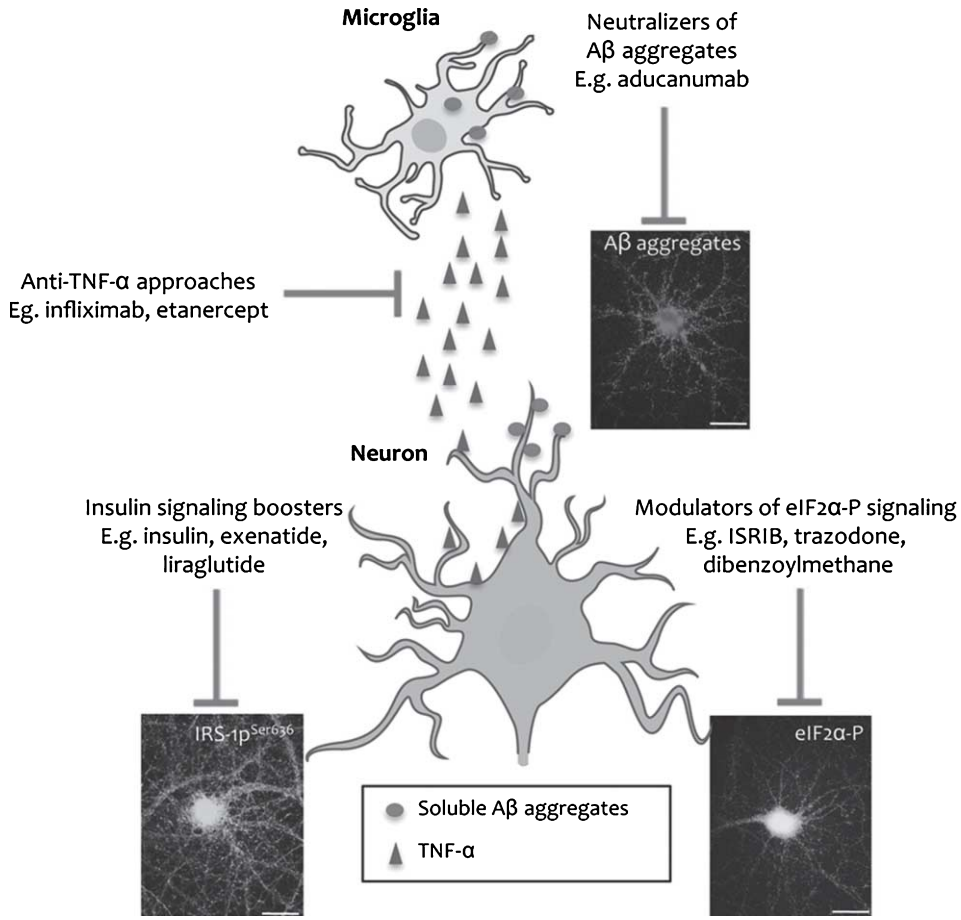

Fig. 1. Cellular basis of brain metabolic dysfunction in AD and potential therapeutic strategies. Brain accumulation of soluble Aβ aggregates causes increased microglial reactivity, thereby resulting in release of pro-inflammatory cytokines (e.g., TNF-α), which in turn triggers neuronal dysfunction. Soluble Aβ aggregates may further act directly on neuronal synapses to impair neuronal homeostasis. Neuronal stress signaling is characterized by elevated inhibitory phosphorylation of both insulin receptor substrate-1 (IRS-1p^{Ser636}) and eukaryotic translation initiation factor 2α (eIF2α-P). Such orchestrated response underlies brain insulin resistance and synapse impairments in brain regions relevant for memory (hippocampus) and peripheral metabolic control (hypothalamus) in AD, and approaches aimed at targeting these noxious mechanisms have been under investigation. Immunofluorescence images depict cultured rat hippocampal neurons stained for Aβ aggregates (upper right; red), IRS-1p^{Ser636} (bottom left; yellow) or eIF2α-P (bottom right; green) after exposure to soluble Aβ aggregate preparations for 3 hours. Scale bar: 20 μm.

Microglial-derived cytokines enhance AβPP processing, induce tau phosphorylation, and contribute to synapse plasticity impairment in neurons [133, 134]. Pro-inflammatory cytokines have further been implicated in memory deficits and depressive-like symptoms in AD models [109, 135, 136]. Microglial signals may prompt astrocytes to assume neurotoxic phenotypes, further contributing to neuronal damage in AD [137]. Finally, reactive microglia induce synapse loss in AD models by stripping off synapses through a complement-dependent recognition system [138, 139].

Genetic studies have pointed that loss-of-function mutations in the Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), notably expressed in microglia, increase up to 4 times the risk of AD in humans [140, 141]. TREM2 is essential for microglia survival, activation, and phagocytosis [142–145]. TREM2-deficient AD mice had impaired microglial metabolism [146], and failed to activate microglia surrounding plaques and to respond to injury, resulting in increased amyloid burden [144, 145]. It is noteworthy that a very recent study uncovered that, in addition to TREM2, variants of the microglial-expressed genes PLCD2 and ABI3 are associated to either protection or increased risk of AD [142]. Their results implicate innate immunity function in AD, further offering a

genetic basis for the link between brain inflammation and AD.

Although microglial actions can trigger detrimental processes in the brain, they also play fundamental roles to maintain brain homeostasis. They release neurotrophic factors, such as BDNF and IGF-1, to influence neuronal survival and synaptic plasticity [147, 148]. It is thus possible to speculate that loss of proper microglial function could itself be harmful in brains undergoing neurodegeneration. Thus, compounds aimed at keeping microglia in good shape are highly warranted for preclinical and clinical AD testing.

In T2DM, increased levels of pro-inflammatory mediators, especially TNF-α, act in the hypothalamus and in peripheral tissues causing activation of intracellular stress kinases such as c-Jun N-terminal kinase (JNK), IκBα kinase (IKK) and double-stranded RNA-dependent protein kinase (PKR). These kinases trigger serine phosphorylation of IRS-1, thereby blocking downstream actions initiated by insulin. As in insulin-resistant peripheral tissues, TNF-α-induced hippocampal activation of JNK has been described in brains of AD transgenic mouse models, Aβ-injected mice and cynomolgus monkeys, and in postmortem analyses of AD brains [31, 121]. Defective insulin signaling has been shown as a consequence of stress kinase activation in AD brains and in several experimental models of AD, in which it was shown to contribute to memory impairment [31, 99].

Furthermore, involvement of both IKK and PKR has been described in AD-linked insulin signaling dysfunction in hippocampal neurons [31, 99, 109]. Notably, blockade of TNF-α with the neutralizing antibody infliximab or genetic deletion of TNF-α receptor 1 led to improved insulin sensitivity [31], normalization of memory performance, and rescued depressive-like behavior in AD mouse models [109, 135, 136, 149–152]. A role for peripheral TNF-α has further been corroborated by recent findings that systemic infusions of anti-TNF-α antibodies rescue memory and glial reactivity [153]. These results provide additional evidence for a close parallel between inflammation-associated defective brain insulin signaling in AD and chronic inflammation-induced insulin resistance in peripheral tissues.

Neuronal stress signaling and defective proteostasis

Defects in protein homeostasis, or proteostasis, have been recently associated with neuronal malfunction and cognitive impairment in AD. Phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) at serine 51 (eIF2α-P) by stress kinases, including PKR, attenuates general protein synthesis, and its sustained elevation has already been associated to memory impairment in rodents [154–156]. Importantly, increased levels of eIF2α-P were found in the brains of AD patients [99, 157–160], as well as in animal models, including APP/PS1 mice, and Aβ-injected mice and cynomolgus monkeys [109, 157]. Moreover, PKR-dependent eIF2α-P appears to be initiated by TNF-α signaling, ultimately leading to hippocampal synapse loss and memory failure [109].

Suppression of two additional eIF2α kinases, PERK and GCN2, was shown to alleviate AD-linked inhibition of long-term potentiation, and to restore spatial memory impairment in AD transgenic mice by replenishing hippocampal protein synthesis [157]. These results were confirmed by additional studies showing neuroprotective actions of PERK inhibition/ablation in AD models [161, 162]. A recent study demonstrated that metabotropic glutamate receptor-dependent long-term depression, which is also impaired in AD mutant mice, is recovered when PERK activation is suppressed, further indicating that normalization of eIF2α-P levels improves synaptic plasticity [163]. In accordance, activation of PKR and PERK has been reported in postmortem AD brains [159, 164–167], likely suggesting clinical relevance to these experimental findings.

In addition to attenuation of general protein synthesis, sustained eIF2α-P paradoxically leads to the enhanced translation of selective mRNAs, including that of activating transcription factor 4 (ATF4), a repressor of long-term synaptic plasticity and memory that counteracts pro-memory signaling [168, 169]. Increased levels of ATF4 have been found in the brains of AD patients and transgenic mouse models [157, 158, 170], and aberrant translation of ATF4 in axons mediates neurodegeneration in the AD brain [158]. Finally, elevated eIF2α-P has been shown to increase BACE activity and amyloidogenesis in mouse models [171, 172], likely contributing to amyloid build-up in human AD.

Taken together, these results point to novel molecular mechanisms of cognitive decline in AD initiated by metabolic impairments and resulting in defective proteostasis and synaptic function. Findings further suggest that targeting stress kinases and eIF2α-P levels might be interesting future approaches to restore neuronal homeostasis and synaptic function in AD

(Fig. 1). Pharmacological modulators of eIF2α-P actions have now begun to emerge [173–177], and future studies may assess their preclinical potential in AD and other forms of neurodegeneration.

Peripheral metabolic dysfunction and the hypothalamus

The hypothalamus is a brain region with prominent endocrine actions that regulate, among other physiological functions, sleep/wake cycle, body temperature and, importantly, food intake and lipid/carbohydrate metabolism [178]. Neuroendocrine studies have revealed that aberrant pro-inflammatory and stress signaling pathways in the hypothalamus are sufficient to deregulate peripheral metabolism in diabetes/obesity pathophysiology [179]. Hypothalamic nuclei are highly responsive to peripheral signals, such as those mediated by insulin and leptin. However, sustained hyperinsulinemia, typical of metabolic derangements such as obesity and diabetes, were shown to cause signal-resistance in the hypothalamus [179].

Extensive evidence indicates that low-grade inflammation takes core place in the hypothalamus to impair body metabolism. Hypothalamic disturbance is driven at a molecular level by several of the inflammatory pathways mentioned above to mediate peripheral effects of altered metabolism [180].

Overfeeding and obesity cause a nutrient overload that includes an elevation in circulating levels of free fatty acids [181], which, in turn, stimulate ER stress in hypothalamic neurons [182, 183]. In parallel, high free fatty acid levels directly activate toll-like receptors triggering immediate transduction of pro-inflammatory intracellular cascades in hypothalamic neurons [184]. In addition, central and peripheral cytokines appear to contribute to hypothalamic dysfunction [185, 186].

Such orchestrated response, mediated by stress kinases and transcription factors, will result in defective proteostasis, neuronal insulin/leptin resistance and in a transcriptional shift toward a neurotoxic profile [178, 187, 188]. The main outcome is aberrant hypothalamic function and impaired control of body metabolism in obesity [189]. Therefore, mechanisms that actively operate to damage hippocampal/cortical neurons in AD resemble those that mediate central deregulation of body metabolism in obesity. This notion has led to the hypothesis that the hypothalamus might be affected in AD, thereby offering an explanation on why AD patients develop peripheral metabolic impairments, such as insulin resistance and hyperglycemia.

Initial discoveries suggested that the hypothalamus might indeed be a key brain region that presents amyloid plaque pathology, and that hypothalamic dysfunction occurs early in disease [7, 180]. These studies identified amyloid deposits in AD brains [190, 191], and brain imaging studies revealed reduced hypothalamic volume in early AD patients when compared to non-cognitively impaired subjects [192]. Another study described neurodegeneration in the hypothalamus, with shortened dendritic arborization and synapse pathology in early AD patients [193]. In Aβ-injected rats, accumulation of fibrillar aggregates in the hypothalamus was detected up to three weeks after the injection and was accompanied by hypothalamic astrocytosis [194].

Hypothalamic inflammation, ER stress, and insulin resistance were demonstrated in Aβ-injected mouse and cynomolgus monkeys [32]. Hypothalamic dysfunction was associated with development of persistent peripheral glucose intolerance, which was further observed in different AD transgenic mouse models [32, 195, 196]. Blockade of TNF-α mediated signaling pathways or alleviation of ER stress normalized glucose tolerance [32], indicating that diabetes-linked mechanisms may operate in the hypothalamus to impair peripheral metabolism in AD.

In addition to peripheral metabolism deregulation, it is noteworthy that hypothalamic defects could also underlie other non-cognitive aspects of AD. Hypothalamic nuclei responsible for circadian rhythm maintenance are affected in AD patients and animal models [197–201], raising the possibility that impaired neuronal function in the hypothalamus accounts at least partially for sleeping pattern disruption and aggressive behavior. For instance, several studies have now investigated how sleep becomes deregulated in AD [198, 202, 203], and further studies are warranted to investigate whether hypothalamic inflammation could, at least in part, mediate sleep disturbances in AD.

NOVEL GROUNDS FOR AD RESEARCH

Is it established that metabolic dysfunction comprises a risk factor for AD?

A substantial body of evidence supports that brain insulin signaling deregulation in AD could represent

a clinical link between T2DM and dementia [30, 204]. Confirmation of this notion is central for the development of effective approaches in dementia. As not all reports have found significant effects of metabolic impairments in AD, more discussion on whether T2DM/obesity and other metabolic defects are causally linked to dementia is warranted.

A meta-analysis of 12 studies in large cohorts found a mild association between metabolic syndrome (MetS) onset and poorer cognitive performance [205]. When individuals were separated by age, however, a stronger correlation was observed among the younger (<70 years old) rather than elderly patients, who might present other age-related factors masking putative effects. Indeed, patients that suffer from T2DM and/or MetS comprise very heterogeneous populations. They may take different medications, have different lifestyle habits, and often present different comorbidities, and this should be taken into account in such epidemiological studies.

Furthermore, clinical assessment of MetS does not follow unified criteria. With its onset defined by any three out of five parameters, it is very likely that components of MetS have differential impact on cognition and risk of dementia. Accordingly, an association study demonstrated that obesity had a closer relationship to mild cognitive impairment than other MetS factors [206]. Moreover, a significant positive association between fasting plasma insulin levels and cognitive dysfunction has been reported in a Danish MetS study [207]. Therefore, though a connection between impaired body metabolism and dementia has become increasingly clear, clinical and epidemiological analyses should use careful methodologies to isolate principal components associated with AD onset and progression [208].

Additionally, most epidemiological approaches addressing the connection between impaired metabolism and dementia are rather descriptive, and have not been often confirmed by interventional studies [209]. For example, while higher blood glucose levels have been associated to declining memory [23, 210], data from ACCORD (Action to Control Cardiovascular Risk in Diabetes) trial has not shown beneficial effects of managing glycemia or lipidemia on cognition [211, 212]. Conversely, multidomain interventions aimed at reducing risk factors and controlling body metabolism have been proposed as effective means of reducing dementia incidence [43, 44]. A pioneer investigation has already shown that such approach could preserve cognition in at-risk subjects [213], and replication attempts are underway in the FINGER study (NCT01041989) [214]. Future clinical studies and meta-analyses should investigate possible effects that might explain the contrasting observations in the field and should test the propelled hypothesis that lifestyle interventions aimed at improving metabolism could reduce AD risk at later stages of life.

The future of translational research in AD

Eleven decades have passed since AD was initially described [215] and, despite several proposed etiogenic hypotheses, no drug or approach has been shown to effectively reverse or even slow down dementia progression. Moreover, AD remains largely idiopathic, with only a small fraction of the cases explained by familial mutations, and with yet unknown bona fide molecular predictors or diagnostic biomarkers. It is thus not surprising that clinical trials have failed or been halted. Given that this apparently unsuccessful story is not due to lack of interest or research, it tells us that shifts in current AD research pipeline might be required.

AD has been classically viewed as a proteinopathy in which accumulation of Aβ and tau plays significantly roles on synapse and memory dysfunction [216–218]. From the early concept of insoluble plaques as drivers of memory impairment to a more recent and refined concept of neurotoxicity of soluble Aβ species, the amyloid cascade hypothesis has reigned as the most influential paradigm for AD pathogenesis in the past 30 years [219, 220]. Not less important, however, are the notions that non-canonical forms of tau and ApoE4 trigger neuronal dysfunction and memory loss in animal models [170, 221–228], likely accounting for human AD pathophysiology. There is growing indications that soluble Aβ and tau can be secreted, diffuse trans-synaptically, adopt prion-like behaviors in the brain, and impair synaptic plasticity [229–237]. Notably, synapse plasticity and memory deficits triggered by both soluble Aβ or tau appear to depend on interactions with AβPP [225].

Although Aβ levels start to rise decades before initial clinical symptoms appear in AD-linked mutation carriers [40, 238, 239], the underlying causes for increased brain Aβ and tau in sporadic AD remain poorly understood. An attractive hypothesis postulates that accumulation of injuries throughout life may sum up with poor habits and lifestyles to favor AD onset at later stages [10, 240, 241] (Fig. 2). Thus,

midlife metabolic diseases, including obesity and diabetes, as well as traumatic injuries, could create the neurotoxic conditions for gradual increases in amyloid and tau pathologies to take place in sporadic AD. Aβ and tau, in turn, could exacerbate brain dysfunction by acting on neurons, astrocytes and microglia, and promote the neurodegeneration observed in late AD.

On the other hand, individual genetic features, such as single-nucleotide polymorphisms, mutations or alleles, could represent additional predisposition traits to set the pace for AD onset in association with environmental conditions. Genetic studies have recently taken major steps forward with the identification of TREM2 variants as a risk factor for AD [140, 141]. Follow-up discoveries have implicated TREM2 loss-of-function in the impairment of microglial function [142–144], with consequences to neuroinflammation and Aβ clearance, as the pathophysiological underpinnings of increased AD risk.

Resolution offered by molecular biology and genetic studies has substantially increased with advancing technologies and will shed light on genomic variations that affect the risk for AD and other forms of dementia. Exciting news are that US government has supported a large, controlled, clinical study to map genome variations in AD patients [242], and understanding genetic variations in AD has been set as one of the priorities of the newborn UK Dementia Research Institutes.

As most drugs and therapies tested in rodent models that advanced into clinical trials had disappointing outcomes, history tells us that strategies that were developed based on single assumptions for AD pathogenesis have been misleading, and that early intervention are key to success. Thus, the future of AD research may benefit from 1) improving AD modeling, taking sporadic variables and human-specific traits into consideration; 2) developing efficient diagnostic tools to detect at-risk cohorts as early as possible; and 3) testing combination therapies that target more than a single aspect of disease.

In this context, animal models of AD have recently taken major leaps forward by ongoing attempts to develop non-human primate models [243, 244], which likely better resemble human pathology, and by next-generation AβPP knock-in models [245, 246] that are less prone to neurotoxicity by non-Aβ fragments of AβPP. Still, the field demands future models that are less based on familial AD mutations and that comprise more features of human AD, including neurofibrillary tangles. Second, it is imperative that the complex nature of AD be discriminated by better diagnostic and prognostic biomarkers. Recent discoveries have raised interest and excitement, and a combination of imaging, neuropsychology, and fluid biomarkers might result in more accurate tracking of AD onset and progression [247–249]. Lastly, although reigning Aβ-targeting approaches should not be completely put off the game, there is an exciting trend to move forward with preclinical and clinical testing of combination therapies, which might likely yield more favorable results in large trials.

Repurposing drugs to accelerate disease targeting

Drug development targeting the central nervous system has traditionally high failure rates. For instance, the approval likelihood of new AD drugs between 2002 and 2012 reached only 0.4%, whereas cancer and cardiovascular drugs hit the approval rate of 6.7%, and 7.1%, respectively [12, 250]. Given the urgent need to combat the global burden of AD, policymakers and science leaders have gathered efforts to find ways to effectively treat or prevent AD by 2025 [251]. Nonetheless, if one considers the traditional pipeline from basic research to ultimate clinical testing, it is inevitable to realize the long road until a novel treatment can be labeled as safe and effective for any human disease. Therefore, strategies aimed at repurposing already marketed drugs become an interesting option to accelerate drug discovery for AD and other diseases [252].

The abundant body of data indicating that anti-diabetic compounds could be neuroprotective in preclinical AD studies and in pilot clinical trials has fostered clinical trials in larger cohorts [24, 29]. Insulin, the most well-known anti-diabetic compound, has advanced to clinical trial aimed at determining whether mild-to-moderate AD patients may present memory benefits with continued intranasal delivery (SNIFF; clinical trial ID NCT01767909). This investigation has received significant support after demonstration that intranasal insulin enhances memory in non-cognitively impaired and early AD subjects, and that insulin is neuroprotective against AD-related synapse loss [31, 109, 110, 242]. Exenatide and liraglutide, two compounds already labeled for T2DM management, have advanced into initial clinical trials (NCT01255163 and NCT01843075, respectively) after substantial preclinical investigation [26, 109, 253–257].

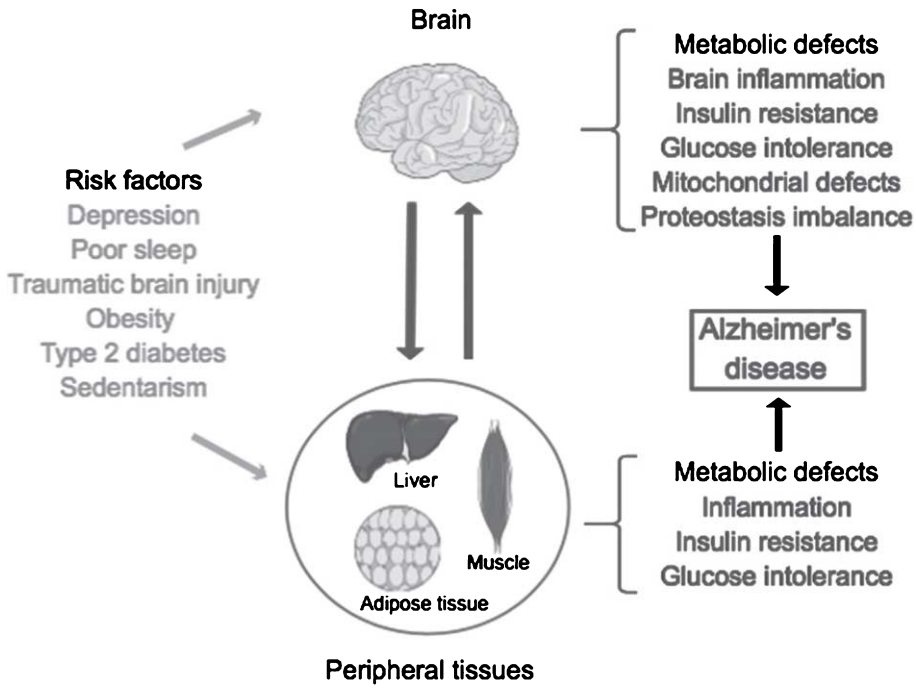

Fig. 2. Risk factors and metabolic defects in AD. Several life conditions have been associated with an increased risk of developing AD. Although some of these disorders, including depression and traumatic brain injury, have been primarily linked to changes in the brain, emerging evidence indicates they might also impact peripheral tissues, such as liver, skeletal muscle, and the adipose tissue. These diseases could also harm additional organs, including pancreas and the gut. In addition, midlife obesity, type 2 diabetes, sedentarism, and poor sleeping habits appear to negatively affect both the brain and periphery. The deleterious impact of such conditions may result in the metabolic defects that favor the onset of AD, including inflammation and insulin resistance (in brain and periphery), and defective mitochondrial function and cellular proteostasis in the brain. Furthermore, improper bidirectional communication between the brain and peripheral tissues through neurotransmitters, hormones, and cytokines might contribute to originate the pathophysiological features of AD. Reducing the metabolic impact of AD risk factors might be key to reducing the number of new cases of dementia in the future.

Given the support for a role of neuroinflammation in AD pathogenesis, it is tempting to hypothesize that anti-inflammatory approaches could also represent effective therapeutics in AD. Results from clinical trials, nonetheless, have been contradictory. Although lifelong use of non-steroidal anti-inflammatory drugs (NSAIDs) was associated with reduced risk of developing AD [258], clinical trials, unfortunately, did not reveal beneficial outcomes for AD patients [259, 260].

Studies with aspirin, nimesulide, ibuprofen, rosiglitazone, and pioglitazone, for instance, have not shown positive effects in randomized clinical trials so far [259, 261–264]. A more detailed investigation in one clinical trial, though, has revealed that naproxen effect vary depending on the stage of the disease. It accelerated AD pathology on later stages of the disease, while it reduced AD risk on preclinical stages [265]. This dual effect of NSAID on AD depending on the disease stage possibly mirrors pleiotropic roles of microglia on the disease. Such apparently disappointing results coming from NSAIDs could be due to the fact that anti-inflammatory agents target generic rather than specific neuroinflammatory components in AD. Thus, future studies may reveal effects of labeled drugs on more refined targets, including microglial modulators, hopefully resulting in more effective strategies for AD therapy. Additionally, it is likely that chronic low-grade inflammation takes place in the brain to cause abnormal elevation of Aβ, tau and neuronal dysfunction and long before initial symptoms emerge in AD. Thus, preventing pro-inflammatory conditions and treating inflammation as early as possible need to be tested as potential ways of slowing AD progression.

Fostering tests on repurposed drugs could be key to accelerate disease targeting pipeline in AD. Table 1 summarizes the current depth of pre-clinical and clinical evidence for drugs that could be repurposed for AD treatment, thus representing new hopes for treating dementia. Advancing on the therapeutic pipeline, it will be now needed to determine whether promising

Table 1
Repurposing drugs for AD therapy: current preclinical and clinical evidence from metabolism-targeting approaches

Mechanism of action	Clinical application	Compounds	Findings from animal models	Clinical evidence
Anti-inflammatory (anti-TNF-α monoclonal antibodies)	Rheumatoid arthritis	Infliximab, etanercept	– rescue neuronal damage and memory impairment in mice [109, 153, 266]	– no large interventional study yet completed in AD patients to assess cognitive outcomes – a pilot study with etanercept has shown positive trends for improved cognition and behavior [267]. Larger trials are warranted to confirm these effects
Anti-inflammatory (NSAIDs)	Inflammatory conditions, pain relief	nimesulide, naproxen, ibuprofen	– reduce brain Aβ prevent impairments in synaptic plasticity and memory in mice [268–270]	– prolonged use of NSAIDs in midlife appears to reduce AD risk at later stages [258] – aspirin, nimesulide and ibuprofen did not result in positive outcomes in clinical trials [259, 261, 264] – Naproxen may reduce AD risk in non-cognitively impaired subjects, but may accelerate AD pathology in patients
Hormone	Types 1 and 2 Diabetes Mellitus	Insulin	– promotes neurogenesis, synaptogenesis, synapse plasticity and memory [271–276]	– improves memory in healthy or cognitively impaired elderly humans [116], and in early AD patients [25, 277, 278] – intranasal insulin has been tested in the SNIFF clinical trial (NCT01767909)
PPARγ agonists	T2DM	Rosiglitazone, pioglitazone	– reduce brain Aβ burden [279], and improve cognition in AD mouse models [280–282]	– no positive outcomes on cognition in AD patients as monotherapy [283] or in combination with AChE inhibitors [263]
Incretins	T2DM	Exenatide, liraglutide	– promote synaptic plasticity and neurogenesis in healthy mice [284, 285], and reduce Aβ burden and memory impairment in AD mice [31, 109, 254, 286] – liraglutide reduced brain stress signaling in a pilot study in Aβ-injected nonhuman primates [109]	– Both compounds are currently under Phase II clinical trials in AD (NCT01255163 and NCT01843075)

modulators of metabolism could indeed represent disease-modifying approaches in AD.

CONCLUDING REMARKS

Knowledge on the complex nature of AD pathophysiology has considerably evolved over the past decades, even though this gain of information has not translated into effective therapies yet. Accumulating observations have implicated metabolic defects, including dyshomeostasis of glucose metabolism, insulin resistance, and disturbed proteostasis, in the course of AD pathogenesis. Impaired metabolism may arise from a combination of genetic and environmental components to increase the risk of AD development, and could further drive cognitive and non-cognitive symptoms, and neurodegeneration.

Considering AD as a metabolic disease and understanding the mechanistic links among AD, obesity, and T2DM could be helpful steps toward developing effective strategies for AD prevention and treatment. Repurposing agents already approved for

the treatment of metabolic disorders may have clinical relevance for AD, as they have already been through preclinical toxicology assessments, human safety, tolerability, and pharmacokinetic assessments. Some clinical trials are now underway and conclusive results might be available in the upcoming years. There is growing agreement that combination therapies might yield more effective results in AD. In this scenario, targeting metabolic impairments might open new avenues to develop alternative therapeutic strategies with higher chances of success.

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Targeting Insulin for Alzheimer's Disease: Mechanisms, Status and Potential Directions

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Abstract. Insulin resistance can occur when the body is unable to respond to insulin even in excess. In the brain, insulin manages glucose metabolism in regions such as the hippocampus and plays a key role in directly regulating ERK, a kinase required for the type of memory compromised in early Alzheimer's disease (AD). Human imaging studies show that brain glucose utilization declines with age and is notably impaired in subjects with early AD. Likewise, animal models of AD or insulin resistance, or both, demonstrate that dysfunctional insulin signaling and insulin resistance in the brain have reciprocity with neuroinflammation and aberrant accumulation of amyloid-β (Aβ), pathological hallmarks in AD. As such, the association between brain insulin activity and AD has led to clinical trials testing the efficacy of insulin and insulin-sensitizing drugs to intervene in AD. Based on recent inquiries to ClinicalTrials.gov, we evaluated thirty-three clinical studies related to AD and insulin. The search filtered for interventional clinical trials to test FDA-approved drugs or substances that impinge upon the insulin signaling pathway. Insulin, metformin, and thiazolidinediones were the three main interventions assessed. Overall, these strategies are expected to negate the effects of brain insulin resistance by targeting insulin signaling pathways involved in neuroinflammation, metabolic homeostasis, synaptic functional and structural integrity. The goal of this review is to provide an update on insulin and ERK signaling in relation to memory, its decline in early AD, and provide an overview of clinical trials related to insulin for early AD intervention.

Keywords: Alzheimer's disease, animal model, clinical trials, ERK, insulin resistance, learning and memory, metabolism, mitochondria, PPARγ

INTRODUCTION

Temporal-parietal networks, including the hippocampus, that underlie episodic memory are functionally and structurally compromised in the earliest stages of Alzheimer's disease (AD) [1, 2] and encompass key diagnostic criteria (atrophy, hypometabolism, amyloid, and tau pathology) defining AD staging [3, 4]. The high glucose demand and insulin sensitivity of the hippocampus places it at particular risk for insulin resistance that is quintessential to aging and age-related disease states such as AD [5–7]. Understanding the molecular processes by which insulin contributes to hippocampal learning and memory and how these break down with aging and disease driving conversion to AD, may facilitate the application of therapeutics with disease-modifying efficacy for early (preclinical) AD [8, 9].

The episodic memory deficits of early AD are thought to result from aberrant amyloid-β (Aβ) accumulation and synaptic toxicity leading to dysregulation of a variety of signaling cascades. In the

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hippocampus, ERK (extracellular signal-regulated kinase mitogen activated protein kinase) is a central integrator for plasticity and memory [10, 11]. In this review, we focus on how insulin resistance may influence early AD memory impairment through the role of insulin signaling in hippocampal learning and memory. This review will address the relationships between the insulin and ERK signaling cascades as they relate to learning and memory and review recent and ongoing clinical trials targeting insulin and insulin signaling in AD with a discussion of potential new therapeutic considerations and directions.

Insulin signaling

Insulin is the predominant mediator of metabolic homeostasis by regulating glucose, energy, and lipids [12, 13]. In the periphery, the pancreas releases insulin with an increased presence of glucose and stimulates necessary cells to take up glucose for ATP production in the mitochondria. In the brain, insulin manages glucose metabolism in regions such as the hippocampus. Insulin also regulates development, liver gluconeogenesis, fatty acid synthesis, and mitogenesis [14, 15]. Insulin signals through its cell surface receptor tyrosine kinase that autophosphorylates and recruits adaptor proteins such as insulin receptor substrates 1 and 2 (IRS1, IRS2) [16] to initiate pleotropic actions through diverse signaling pathways with ERK serving as a convergence point (Fig. 1).

Insulin resistance and metabolic stress

The molecular mechanisms and physiological consequences of insulin resistance have been extensively studied [17, 18]. In brief, insulin resistance induces metabolic stress that manifests as altered mitochondrial function and chronic inflammation that further exacerbate metabolic homeostasis in part through lipid [19] and Aβ metabolism [20], significant risk factors for AD (https://www.alz.org/documents_custom/2017-facts-and-figures.pdf). These perturbations manifest as an inability to properly respond to insulin (insulin resistance) that is typified by hyperinsulinemia, hyperglycemia, and hyperlipidemia [21, 22] which can predispose for the metabolic syndrome and diseases such as type 2 diabetes, obesity, cardiovascular disease, chronic inflammation, and AD [23].

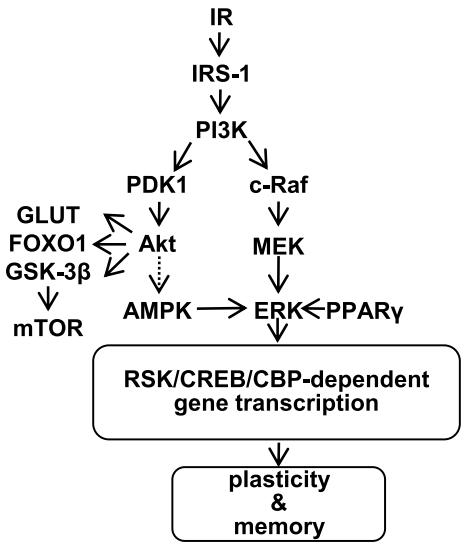

Fig. 1. Insulin signaling converges upon the ERK cascade for learning and memory in the hippocampus. Insulin signaling axis affects mediators of glucose utilization (GLUT, GSK-3β), mitochondrial function (FOXO1), and energy metabolism (mTOR, AMPK) to support hippocampal integrity. Insulin signaling converges on ERK and memory through RSK/CREB/CBP-dependent gene transcription. ERK, extracellular signal regulated kinase; GLUT, glucose transporter; GSK-3β, glycogen synthase kinase 3 beta; FOXO1, forkhead box protein 01; mTOR, mechanistic target of rapamycin; AMPK, AMP-activated protein kinase; RSK, ribosomal S6 kinase; CREB; cAMP response element binding; CBP, CREB binding protein.

Insulin and ERK in learning and memory

Insulin is secreted from the β-cells of the pancreas to maintain glucose homeostasis in the periphery. Insulin signaling in the brain and its impact on metabolism and function are similar to those established in the periphery [24]. However, insulin plays a profound role in brain function including metabolic homeostasis and cognition. Insulin signaling in the brain impinges upon the ERK signal transduction cascade for hippocampal synaptic plasticity, learning, and memory through the maintenance of homeostatic redox, inflammatory, lipid, and glucose metabolism within neural networks. Several lines of evidence support the model that excess Aβ mediates the association between insulin resistance and cognitive impairment in early AD and, due to the heightened metabolic and energy needs of this brain region, the hippocampus is particularly vulnerable to these processes in early AD [6, 25, 26].

In the hippocampus, the ERK cascade is essential to the induction and maintenance of long term potentiation (LTP) and memory consolidation as it

converges with a number of other signaling cascades, including CaMKII, PKA, and PKC [10, 27, 28]. Further, ERK can be activated by a number of receptors and second messenger systems involved in cell homeostasis including the insulin receptor, a receptor tyrosine kinase, that couples to ERK via PI3K [29]. In the canonical ERK pathway [10, 28], peptide growth factors and hormones (e.g., insulin) bind to their corresponding receptor tyrosine kinases leading to several phosphorylation and translocation events that lead to guanine nucleotide exchange factor activation and GDP-GTP exchange on the small G protein Ras. Active Ras then recruits the serine/threonine kinase Raf to the cell membrane, where it is activated and phosphorylates the dual specificity kinase MEK. MEK then binds to and dually phosphorylates ERK to initiate a number of downstream effects, including the phosphorylation-dependent activation of kinases and transcription factors that induce memory consolidation-dependent gene expression [30–32], and the facilitation of protein synthesis and the remodeling or stabilization of dendritic spines [10, 33–35] necessary for LTP and memory (Fig. 1).

The requirement for ERK activation in LTP and hippocampus-dependent learning was established by using MEK inhibitors such as PD098059, SL327, or U0126 to block these processes [30, 36, 37]. More detailed analyses found that stimulation of the Schaffer collateral inputs to the hippocampal CA1 region selectively activated the p42 isoform of ERK [38, 39], and that the p42 isoform (heretofore referred to as 'ERK2') is activated following training in a hippocampus-dependent cognitive task [36]. Furthermore, knockout mice in which the p44 isoform of ERK (ERK1) was deleted do not exhibit hippocampal LTP deficits or impairment in hippocampus-dependent memory formation [40]. Since ERK is fundamental to cellular homeostasis, it poses significant challenges as a therapeutic target. It is therefore imperative that we apply our understanding of the molecular processes of learning and memory to early AD pathophysiology to identify components of this highly integrated signaling network as viable therapeutic targets.

ALZHEIMER'S DISEASE

Pathogenesis

The aging and dementia research community has made significant progress in the past 25 years by identifying causative genes and risk factors as well as characterizing the clinical and pathologic features of AD [41]. These findings led to a reconceptualization of AD as having a long preclinical phase during which significant pathology is present prior to clinically significant cognitive impairment [8, 9, 42, 43]. This preclinical phase is followed by two additional stages termed MCI (mild cognitive impairment) due to AD and dementia due to AD that now have new diagnostic and biomarker criteria [9, 43–47]. The pathological hallmarks of AD are accumulation of Aβ in plaques and hyperphosphorylated tau in neurofibrillary tangles [45]. However, in the two decades, oligomeric forms of Aβ have been implicated as a culprit initiating AD pathophysiology and an emerging concept is that while Aβ oligomers trigger disease, misfolded tau is requisite for full neurotoxicity [45, 48]. In any case, these misfolded proteins have a neuroinflammatory and metabolic dyshomeostasis component that interrelates with insulin resistance and feed forward exacerbation of AD.

Risk factors

AD cases are broadly categorized as either inherited early-onset or sporadic late-onset AD (LOAD), with the overwhelming majority (90–95% or more) of cases qualifying as LOAD. Early-onset, familial AD describes individuals who develop AD before the age of 65 due to the inheritance of autosomal dominant gene mutations with symptoms occasionally appearing as early as 30 years of age. The genes causative for developing AD are the autosomal dominant mutations in APP, presenilin 1 (PS-1) and presenilin 2 (PS-2). The risk of developing LOAD is thought to arise due to interactions between inherited and environmental risk factors that function in an age-dependent manner [49, 50]. Inheritance of APOE4 is the most significant risk factor for LOAD [51]. Individuals who carry two copies of the ε4 allele have a higher risk than those who carry only one copy, and both groups have a higher risk than those who carry only the ε2 or ε3 forms [52]. While the exact reason for increased risk is unclear, it is known that APOE enhances proteolytic clearance of Aβ and that the ε4 variant is associated with less efficient clearance than the ε2 or ε3 isoforms [53]. It is noteworthy that being an APOE4 carrier does not guarantee development of AD, and lacking APOE4 is not preventative, suggesting that factors in addition to Aβ burden have significant impact on LOAD susceptibility.

While APOE is a critical contributor to genetic risk, more than a third of AD cases do not carry any APOE4 alleles, and as LOAD heritability has been estimated at ∼80% [54], this suggests much of the heritability has not yet been characterized. These statistics prompted many genome-wide (GWAS) and rare variant association studies to characterize the mosaic of genetic contributors to LOAD. The most recent of these studies identified approximately 20 genes with common variants contributing to LOAD risk (for a comprehensive review, see [55]). The proposed function/contribution of these 20 genes are categorized into the following biological processes or pathways: cholesterol and lipid metabolism, immune and complement function, inflammatory response, synaptic vesicle and receptor endocytosis, general endocytosis and cargo sorting, synaptic function, cytoskeletal and axonal transport function, and tau pathology. As will become evident in ensuing sections, insulin resistance is easily tied to several GWAS hits via the proposed influence on inflammatory and immune function, metabolism, and synaptic function and integrity.

A variety of non-genetic risk factors have been identified for insulin resistance, and therefore AD, including stress, obesity, arthritis, brain injury, diet, sleep, education, and physical and social activity [56, 57]. For example, cardiovascular disease, as exemplified by high blood pressure, heart disease, stroke, and high cholesterol [58, 59], are significant risk factors for LOAD as vascular damage is increasingly appreciated as contributing to the cognitive impairment profile associated with early AD [60–62]. More recently, chronic metabolic disorders such as glucoregulatory abnormalities and insulin resistance that precludes type 2 diabetes have been recognized as significantly contributing to LOAD risk [9, 63–66]. Chronic inflammation is a common denominator in many of these conditions that contribute to risk for insulin resistance as well as AD dementia [67–69]. Furthermore, a normal consequence of aging is loss of insulin sensitivity that can progress to insulin resistance depending on comorbid lifestyle factors [70]. That traumatic brain injury stimulates Aβ production and causes insulin resistance where insulin sensitizers provide symptomatic relief [57, 71] supports the notion established in animal models for AD-like amyloidosis that aberrant production and accumulation of Aβ can induce insulin resistance that contributes to cognitive deficits [72, 73]. Human genetics, including ethnicity, also play a key role in contributing to insulin resistance [41, 74] and therefore to AD risk. Nonetheless, the poorly understood mechanisms underlying the combined risk of genetic and environmental factors are considered to underlie the majority of LOAD [75].

Insulin resistance in Alzheimer's disease

Type 2 diabetes is a chronic metabolic disorder characterized by peripheral insulin resistance, hyperglycemia, and hyperinsulinemia [76, 77]. Epidemiological studies consistently link type 2 diabetes, as well as intermediate stages of insulin resistance, with increased risk of developing AD [59, 63, 78–85]. Type 2 diabetics have up to a 65% increased risk of developing AD [63]. Furthermore, clinical studies have found evidence for central insulin resistance in the AD brain [86, 87] as well as dysregulated glucose metabolism and peripheral insulin resistance in AD patients [88]. Thus, impaired insulin signaling is strongly linked to AD pathology [89, 90].

The link between these two disease states may be based upon the role of insulin in brain metabolism and plasticity [24]. Insulin receptors are widely distributed in brain regions known to be involved in memory function, including high concentrations at synapses in the hippocampus and amygdala, and moderate expression in cortex and cerebellum [91]. Insulin readily crosses the blood-brain barrier in order to regulate glucose utilization [92] and this process influences amyloid, neuronal survival, energy metabolism, and neural network plasticity [93–97]. Furthermore, acute insulin administration improves memory in both humans and rodents [95, 98, 99], and disruption of CNS insulin signaling leads to cognitive deficits in rodents [83, 100]. Subsequent studies in animal models for insulin resistance, AD, or both, have established that insulin resistance exacerbates Aβ and tau phenotypes including enhanced Aβ 42/40 ratio, total tau, and hyperphosphorylated tau [101–108] and AD amyloidosis models exhibit insulin resistance [72, 109].

Insulin sensitizers prescribed for diabetes include the TZDs rosiglitazone (RSG) and pioglitazone (PIO) that target the nuclear receptor and transcription factor, peroxisome proliferator receptor-activator-γ (PPARγ), and the biguanide Metformin (MFM) that targets AMP-activated kinase (AMPK). Activation of PPARγ with RSG or PIO leads to a gene repertoire that promotes insulin sensitivity [110–113]. MFM, an AMPK agonist, is the first line treatment for normalizing insulin resistance in type 2 diabetes [114, 115].

Diabetes treatments and risk of heart failure

Diabetes is a chronic, progressively worsening disease associated with a variety of microvascular and macrovascular complications. Cardiovascular disease is the main cause of death in these patients and many people diagnosed with type 2 diabetes are comorbid for cardiovascular disease and even congestive heart failure. As such, the primary treatment goal in type 2 diabetes is restoration and maintenance of normoglycemia to prevent cardiovascular disease and in reducing diabetes-related end-organ disease [116]. The range of therapeutic options has been extended with the introduction of TZDs used as monotherapy or in combination with oral hypoglycemic drugs or insulin are effective in lowering blood glucose to achieve glycemic goals. There is substantial interest in whether these agents reduce or modify risk of cardiovascular disease through a wide range of PPARγ-mediated effects on the cardiovascular system, in addition to their recognized efficacy as glucose-lowering drugs to treat type 2 diabetes [117–119].

Edema is a recognized side effect of these drugs, particularly when combined with insulin making it important to be cognizant of the risk of congestive heart failure when TZDs are used in patients with type 2 diabetes. Multiple retrospective clinical reviews have been performed to ascertain the cardiovascular safety of TZDs in patients with diabetes. In general, TZDs for type 2 diabetes decreases one's risk for death overall as well as from heart failure or cardiovascular disease compared to no treatment [120].

The beneficial effects of TZDs on glycemia and cardiovascular risk factors have made them attractive agents in patients with type 2 diabetes who are at high risk for cardiovascular disease. There is a growing recognition, however, that edema can occur. Because people with diabetes are at increased risk for cardiovascular disease and many have preexisting heart disease, the edema that sometimes accompanies the use of a TZD can be cause for concern, as it may be a harbinger or sign of congestive heart failure.

In the absence of a properly powered and appropriately designed clinical trial to specifically address the safety and possible benefit of anti-hyperglycemic drugs on the development and progression of heart failure, this question will remain outstanding in the diabetes field as well as whether there is cause for concern in treating AD with TZDs and other drugs for glycemic control.

Insulin resistance-mediated neuroinflammation

Neuroinflammation as well as dysregulated mitochondrial function and metabolic dyshomeostasis are common to several chronic diseases, such as obesity, type 2 diabetes, metabolic syndrome, cancer, and cardiovascular diseases [121]. Chronic elevated blood glucose that accompanies insulin resistance promotes inflammatory responses from the peripheral innate immune system to further exacerbate insulin resistance: IFNs, TNF-α, IL1-β, and IL-6. This scenario is thought to create an inflammatory milieu that exacerbates insulin resistance via feedback inhibition of the insulin receptor and, through a feed-forward mechanism, to perturb mitochondrial function, induce reactive oxygen species production [13, 122–125] and recruitment of NFκ-B-inducing kinase (NIK) [126, 127].

Neuroinflammation is precipitated both by peripheral immune cells and proinflammatory cytokines that cross the blood-brain barrier in addition to inflammatory cytokine production within the brain innate immune system as a result of local toxic insults [128–131]. Dysregulation of these important homeostatic processes are also hallmarks of LOAD [78, 87, 132]. For example, soluble misfolded Aβ leads to neuroinflammatory cytokine production (e.g., TNF-α) through a NIK-dependent pathway [133, 134], suggesting that Aβ-mediated CNS inflammatory responses contribute to brain insulin resistance [87] in addition to its established role in synaptic toxicity during early AD [135-137]. In addition, insulin resistance and accompanying inflammation compromises mitochondrial function [123, 124] to further exacerbate glucose and lipid dyshomeostasis through compromise of TCA cycle, oxidative phosphorylation, ATP synthesis and transport, solute and protein transport, reduction-oxidation (redox) balance, in addition to homeostatic anabolism and catabolism [15]. Thus, current thinking has it that Aβ- and insulin resistance-mediated neuroinflammation further exacerbates neurodegeneration, insulin resistance, mitochondrial and metabolic dysfunction are interrelated and drive precipitous AD cognitive decline [138].

Epidemiology of insulin resistance and Alzheimer's disease risk

Several clinical studies have tested the efficacy of insulin sensitizing TZDs (RSG and PIO) in AD patients, mostly reporting failure to prevent or

improve cognitive and functional decline in those suffering moderate to advanced AD. In contrast, those AD pilot clinical trials assessing RSG or PIO in subjects with early stage disease have found cognitive benefit in subjects comorbid for insulin resistance or those that are APOE4-negative [139–144]. While these studies have remained unreplicated in larger study designs, the numerous positive outcomes in animal models for preclinical AD/MCI suggest that exploratory studies of TZDs as a potential preventative remain warranted (e.g., the TOMORROW study: NCT01931566).

The concept that TZD treatment for AD would be most efficacious prior to severe AD is further supported by a recent large epidemiological study that revealed a sizable protective effect of long-term PIO treatment in type 2 diabetes [145]. Using observational data from 2004–2010, Heneka and colleagues analyzed the association of insulin sensitizer therapy and the incidence of dementia in a prospective cohort study of 145,928 patients categorized as nondiabetics, diabetics without PIO, diabetics with prescriptions of <8 calendar quarters of PIO, and diabetics with ≥8 quarters of PIO. Using Cox proportional hazard models Heneka et al. (2015) explored the relative risk of dementia incidence dependent on PIO use adjusted for sex, age, use of RSG or MFM, and cardiovascular comorbidities. Their analysis confirmed previous observations that patients with type 2 diabetes showed a higher risk of developing dementia [7]. Importantly, PIO treatment was associated with a significantly reduced incidence of dementia in type 2 diabetes patients over the observation period. This protection was dependent on the duration of PIO therapy and increased with each quarter of prescription. RSG showed a similar trend. While this analysis does not causatively link PIO therapy with alleviating AD clinical progression, the findings support prospective clinical trials with type 2 diabetes patients and nondiabetics, possibly with and without insulin resistance comorbidity, to evaluate possible neuroprotective effect.

ALZHEIMER'S DISEASE ANIMAL MODELS

AD pathology modelled in mice

The criteria for staging and diagnosis of AD were recently revised to reflect new knowledge regarding biomarker profiles for the disease. With refinement of clinical staging comes the realization that many of the mechanistically-conceived animal models are more representative of preclinical AD and possibly MCI [146–148]. These observations are consistent with results from a handful of AD pilot clinical trials using the insulin sensitizing TZDs (RSG, PIO) that target PPARγ mainly provide cognitive benefit during early stage AD; generally in conjunction with insulin resistance or diabetes [139–144]. Thus, animal models may provide vital predictive power to future clinical trials by informing upon the disease stage profile that best matches a particular therapeutic intervention [23].

While genetic mouse models of AD do not fully recapitulate the full spectrum of the human disease, these models have been invaluable for the study of preclinical and early AD mechanisms as well as testing potential therapeutic strategies. Since the known gene mutations that cause familial AD produce congruent pathology observed in LOAD, many of these models express human transgenes containing mutations associated with familial AD [149–151]. As such, there are a number of transgenic mouse models of AD, including those that lead to aberrant processing and accumulation of Aβ (Tg2576, PDAPP, presenilin conditional KO/APP, PS1/APP, CRND8, PGDF-APPSW) and mutant tau for neurofibrillary tangle formation (P301S, rTg4510, 3xTg-AD, 5xTg-AD). All of these models exhibit age-dependent cognitive decline in a variety of hippocampus-dependent neurobehavioral paradigms [152] that recapitulate during the earliest stages of AD [4, 9, 44].

Here we discuss studies using the Tg2576 mouse model for AD that helped elucidate how these processes contribute to AD hippocampal dysfunction and cognitive impairment [23, 72, 73, 109, 153–158]. While the extant literature contains several examples of alternative models for AD and insulin resistance [159–163], the most comprehensive body of work on this subject is encompassed by Tg2576 [23, 72, 73, 109, 153–158]. Thus, we will next discuss this model in terms of mechanisms underlying cognitive deficits due to aberrant accumulation of Aβ and comorbid insulin resistance prior to the onset of overt neuropathology.

Tg2576 mice express human APP_{695} containing the familial 'Swedish' mutation (Lys670Asn670, Met671Leu671) [164] that leads to elevated Aβ by 2 MO (months old), and exhibit age-dependent accumulation of misfolded Aβ leading to plaque formation by ∼12 MO. Accumulation of misfolded oligomeric forms of soluble Aβ is believed to be

responsible for the AD-related hippocampal synaptic and cognitive dysfunction that manifests at 5-6 MO [154, 156, 164–169]. While Tg2576 do not form neurofibrillary tangles, they do accumulate oligomeric tau by 5 MO, which recent work has suggested represents the toxic species that furthers cognitive decline [170, 171]. Tg2576 also do not portray overt neurodegeneration yet Tg2576 exhibit synapse and volume loss in the hippocampus [147, 172] which is considered one of the best correlates to cognitive impairment of early, and possibly preclinical, AD [8, 9, 44, 173]. Furthermore, Tg2576 possess dysregulated hippocampal ERK [174], impaired recognition memory, and deficits in ERK-dependent episodic, associative, and spatial learning and memory [165, 166, 168, 175]; all correlates of the cognitive deficits identified in early AD [176–178]. Based on these observations and the current biomarker criteria [9, 44], Tg2576 and related models approximate the pathological transition from preclinical AD to MCI and are well-suited to pursue therapeutic interventions to delay onset of memory dysfunction in subjects with insulin resistance rather than reversing cognitive and neuronal loss due to the advanced neuropathology of AD.

Insulin resistance profiles and therapeutic mechanisms

Early studies found that diet-induced insulin resistance promotes AD pathology and exacerbates cognitive deficits in Tg2576 [106, 179]. Coincident work revealed that Tg2576 manifest peripheral insulin resistance in an age-dependent manner in the absence of diet manipulations [73]. Our evaluation of Tg2576 for insulin resistance assessed peripheral insulin and glucose regulation by directly measuring serum insulin and glucose as well as performing the fasting glucose tolerance test [72, 109]. These measures showed that 5 MO Tg2576 are normoglycemic and normoinsulinemic with the emergence of peripheral insulin resistance and hyperinsulinemia by 9 MO. We previously reported that 9 MO Tg2576 respond to cognitive enhancement with the PPARγ agonist and insulin sensitizer RSG while 13 MO Tg2576 are unresponsive to this intervention [72, 153]. This suggests that a different aspect of the insulin signaling pathway might be exploited during more severe cognitive impairment.

Activation of PPARγ with RSG in the Tg2576 mouse model of AD facilitates hippocampus-dependent memory consolidation and leads to induction of genes regulated by promoters with PPREs (PPARγ response elements) and CREs (cyclic-AMP response elements) [153], the latter of which are prototypically regulated by CREB (cyclic AMP response element binding protein) and CBP (CREB binding protein) transcription factors. Upstream from these memory consolidation gene transcription events is the common integrator of insulin signaling pERK, which is recruited to PPARγ during memory consolidation [180] in the hippocampus [10, 28]. MFM, an AMPK agonist, is the first line treatment for normalizing insulin resistance in type 2 diabetes [114, 115] and is a serious contender for clinical intervention in AD [181–183]. In the periphery, AMPK is a key cellular sensor of reduced energy supply and is an additional therapeutic target in diabetes to increase insulin sensitivity. Although little is known of AMPK function and regulation in the CNS, it is implicated in AD and evidence shows that neuronal AMPK regulates ERK [182, 184]. Insulin resistance leads to downregulation of AMPK in the periphery [185] and is a negative regulator of ERK activity in neurons [186] which is consistent with our previous observations of hyperactive ERK in 13 MO Tg2576 hippocampus [174]. In these contexts, it will be important to pursue additional treatment strategies during the age-dependent cognitive decline to fully understand the potential value of AMPK as a therapeutic target. Finally, these observations support our overall hypothesis that proper ERK dynamic range is imperative for proper cognitive performance and determines efficacy of therapeutic interventions that target hippocampal cognitive function.

In vitro and *in vivo*, MFM attenuates AD-like neuropathology by reducing hippocampal tau phosphorylation and one of the tau kinases, c-jun N-terminal kinase [187], while also activating the tau phosphatase mTOR/protein phosphatase 2A in mouse primary neurons [188]. MFM also ameliorates AD-like molecular and neuropathological hallmarks of insulin signaling in neuronal insulin resistance in the Neuro-2a cell line [189]. In addition, MFM reduces BACE1 and, as a consequence, Aβ production in SH-SY5Y-APP neuroblastoma cells, mouse primary cortical neurons, and wild-type mice [190].

In our hands, MFM has a rather specific therapeutic window in that it improves cognitive function between 12–14 MO yet is ineffective when intervention and cognitive testing is performed at earlier ages. Thus, targeting the insulin signaling pathway during early AD cognitive impairment represents a viable therapeutic opportunity based upon empirical evidence gleaned from animal models that insulin

resistance, AD pathology, and cognitive decline are mechanistically interrelated.

Therapeutic windows?

In support of the therapeutic window concept, we have identified three age ranges in Tg2576 that define sensitivity to intervention to improve cognitive function. Suppression of calcineurin activity effectively reversed cognitive deficits in 5 MO Tg2576 but not at older ages [166]. Interestingly, suppression of calcineurin activity improves insulin sensitivity in normoglycemic human subjects but exacerbates insulin resistance in diabetics [191, 192], consistent with our observation that the therapeutic window for calcineurin intervention is in normoglycemic 5MO, but not older, insulin resistant (>8 MO) Tg2576 [72]. These observations may reveal the AD equivalent of the prediabetes state [68] where elevated glucose, in the absence of overt insulin dyshomeostasis, can increase the risk of cognitive decline [193]. Similarly, the observation that 5 MO Tg2576 have upregulated PPARγ whereas it is downregulated in 9 MO Tg2576 delineates the therapeutic window for intervention with PPARγ agonism. Finally, MFM-mediated cognitive enhancement in 13 MO Tg2576 corresponds with hyperactive/hyperphosphorylated ERK as well as down-regulated AMPK. Thus, we propose that disease stage-specific therapeutic windows exist for intervention [23]. This hypothesis is not new and is supported by work using other AD models [194, 195] as well as clinically defined AD stage-specific biomarkers [196–198]. The age-dependent decline in Tg2576 hippocampal cognitive function, progression of insulin-related signaling dysfunction, and mechanistically distinct therapeutic interventions for enhancement of hippocampal cognition with advancing age, provides compelling evidence for disease stage-specific therapeutic windows.

Central insulin resistance, neuroinflammation, and mitochondrial dysfunction

There is much clinical evidence of impaired central insulin sensitivity as well as peripheral insulin resistance in non-diabetic AD brains [86, 87, 199]. However, it is not certain whether these changes occur simultaneously or one before the other. Phosphorylation of insulin signal transduction intermediates is a key regulatory feature of this pathway. Therefore, dysregulated (hyper- or hypo-) phosphorylation of these intermediates is diagnostic of insulin resistance. For example, IRS-1 plays a key role in transmitting signals from its upstream receptors to intracellular PI3K/Akt and ERK pathways (FIG); however, the precise stoichiometry of IRS-1 phosphorylation is governed through feedback and feed forward inhibition exerted by several kinases including ERK2 and mTOR [200–203]. Furthermore, IRS-1 Ser616 phosphorylation is important because it correlates both with amyloid oligomers and episodic memory deficits in MCI and AD [87].

Using Tg2576 and 3xTg-AD mice at ages before and coincident with amyloid deposition, we monitored the total and phosphorylated status of several key components of the insulin signaling pathway [72, 109]. We found evidence that CNS insulin signaling dysregulation precedes the onset of peripheral insulin resistance in both Tg2576 and 3xTg-AD mice. In contrast, alterations in markers of energy homeostasis were detected only after the onset of both central and peripheral insulin deficits. Thus, the common pathology between the two models, Aβ misfolding and accumulation, appears capable of driving CNS insulin signaling dysregulation and peripheral insulin resistance. However, in 3xTg-AD mice, markers for CNS insulin resistance manifested earlier relative to peripheral insulin resistance and progressed more aggressively, suggesting that discrepancies between Tg2576 and 3xTg-AD mice CNS insulin signaling may be the result of the tau pathology developed by 3xTg-AD mice.

Similar studies in which insulin resistance was exacerbated through diet-induced showed down-regulation of insulin receptor signaling in the brain of insulin-resistant Tg2576 mice and is associated with decreased AKT/PKB activity leading to GSK-3 activation [106]. Likewise, alternate AD transgenic models also exhibit insulin pathway perturbation [160, 204–206].

Long after central insulin resistance has ensued, Aβ plaque deposition is observed (>12 MO) Tg2576. This is evidenced in the form of TNF-α, IL-6, and IL-1β positive astrocytes surrounding plaque deposits [207, 208]. The local immune response detected around cortical Aβ deposits in Tg2576 mouse brain is seemingly different to that observed in brains from AD patients. Since the full scope of elevated cytokines found in AD brains are lacking in aged Tg2576 brain, this likely reflects differences in the murine neuroinflammatory process and may help explain why this mouse model for Aβ amyloidosis fails to progress to overt neurodegeneration. Nonetheless, Tg2576 neuroinflammation is

presumed to be triggered by soluble misfolded Aβ as shown *in vitro* [133, 134], further suggesting that Aβ contributes to brain insulin resistance [87] in addition to its established role in synaptic toxicity during early AD [135, 136].

Tg2576 also exhibit mitochondrial protein alterations concomitant with memory impairment and insulin resistance [72, 153]. Cognitive enhancement with PPARγ agonism normalizes many of these proteins critical to energy and metabolism including proteins involved in ATP production, mitochondrial membrane potential and transport dynamics, and redox balance [153]. These findings dovetail with current thinking that neuroinflammation, insulin resistance and mitochondrial dysfunction are interrelated and contribute to the molecular pathogenesis of AD cognitive decline [23, 122, 132].

In summary, studies in animal models for insulin resistance, AD, or both, have established that insulin resistance exacerbates Aβ, and tau phenotypes including enhanced Aβ 42 ratios, total tau, and hyperphosphorylated tau suggesting that AD pathology and impaired insulin signaling form a reciprocal relationship. These and other mechanistic similarities have led the medical community to list insulin resistance as a risk factor for the development of AD.

CURRENT CLINICAL TESTING

As discussed, insulin impinges upon signal transduction pathways that influence synaptic plasticity and memory as well as energy homeostasis and metabolism. Supported by several pilot clinical trials indicating memory and AD biomarker improvement with insulin in AD [86, 88, 98, 209–211], that appears to also depend upon ApOE genotype [96, 199, 211, 212], targeting the insulin signaling pathway in AD has gained favor as an intervention or prevention strategy. Here we summarize and discuss the current clinical trials that either directly deliver insulin to the brain or utilize insulin sensitizers or alternative diet and anti-oxidant strategies to improve cognitive function and biomarker profiles at different stages of AD.

Insulin

Supported by several pilot clinical trials indicating memory and AD biomarker improvement with insulin in AD [86, 88, 98, 209–211], that appears to also depend upon ApOE genotype [96, 199, 211, 212], targeting the insulin signaling pathway in AD has gained favor as an intervention or prevention strategy. Intranasal insulin trials are currently the most common among the clinical trials registered for mild AD or MCI due to AD. Intranasal insulin delivery is considered a safer administration route to systemic insulin administration to elderly non-diabetics due to risk of inadvertent hypoglycemia [213]. Intranasal delivery directs the insulin into the brain, avoiding systemic side-effects. Also, the CSF/serum insulin ratio is lower in insulin resistant individuals compared to insulin sensitive individuals with possible confounding issues regarding insulin transport across the blood-brain barrier. Intranasal insulin addresses this issue by exploiting the CNS access afforded by the nasal passages being contiguous with the olfactory bulb [214].

The SNIFF (Study of Nasal Insulin to Fight Forgetfulness) studies (currently $n = 5$) are interventional double-blind placebo controlled clinical trials to study the effects of insulin and insulin analogs on various AD-related outcome measures in MCI and mild AD, e.g., cognition, glucose metabolism, plasma and CSF biomarkers, daily functioning (Table 1). The studies differ with respect to insulin/insulin analog, dose, treatment duration, and outcome measures. Insulin analogs employed include detemir that has longer half-life and aspart that is recombinant insulin with a single amino acid change to provide faster absorption.

Two SNIFF studies have reported results (ClinicalTrials.gov ID# NCT00438568 and NCT01547169). SNIFF 120 delivered short acting insulin (regular insulin) for 120 days and SNIFF LONG 21 administered long-acting insulin (detemir) for 21 days in randomized, placebo-controlled trials for MCI or AD. Both trials tested the effectiveness of each insulin type for improving memory and daily functioning in comparison to placebo. SNIFF 120 with regular insulin found that 20 IU of insulin improved delayed memory and preserved general cognition in amnestic MCI and mild/moderate AD [98]. SNIFF LONG 21 using detemir found that APOE carrier subjects administered with 40 IU showed significant improvements in memory, and subjects with higher baseline insulin resistance showed more improvement with the 40 IU dose [215].

The SNIFF-Quick study will administer aspart (12 weeks) for effects on cognition and daily function in comparison to placebo (Clinicaltrial.gov ID # NCT02462161). Completion is expected in July 2018. In another study, SNIFF SL120 tests detemir alongside regular insulin in MCI and AD patients

Table 1
Recent clinical trials targeting brain insulin activity in AD and MCI. A recent ClinicalTrials.gov search identified 18 clinical trials related to insulin and insulin-sensitizer therapy. Information gleaned from ClinicalTrials.gov and other sources are summarized. Additional study design details can be found under the ClinicalTrials.gov Identifier. INI, intranasal insulin

Intervention	Title	ClinicalTrials.gov ID #	Start Date MM/YY	End Date MM/YY	PI(s) Sponsor(s)	Status	Reference(s)	Notes/Additional Information	Inclusion Criteria
INI Insulin	Study of Nasal Insulin to Fight Forgetfulness (SNIFF 120)	NCT00438568	06/06	12/11	Suzanne Craft, PhD-University of Washington	completed	https://www.ncbi.nlm.nih.gov/pubmed/21911655	20 IU of insulin improved delayed memory ($p < 0.05$), and both doses of insulin (20 and 40 IU) preserved caregiver-rated functional ability ($p < 0.01$). Both insulin doses also preserved general cognition ($p < 0.05$).	clinical diagnosis of probable AD or has MCI or mild AD, evidence of significant clinical disorder, no diabetes, Ages above 21 or 55–85
INI Insulin	Study of Nasal Insulin to Fight Forgetfulness - Long-acting Insulin Detemir - 21 Days (SNIFF-LONG 21)	NCT01547169	03/11	12/12	Suzanne Craft, PhD-University of Washington	completed	https://www.ncbi.nlm.nih.gov/pubmed/25374101	A dose-finding study in which subjects receive one of two doses of Determir or a placebo (3 weeks).	
INI Aspart	Study of Nasal Insulin to Fight Forgetfulness - Short-Acting Insulin Aspart (SNIFF-Quick)	NCT02462161	05/15	07/18	Suzanne Craft, PhD-Wake Forest University Health Sciences	ongoing		Pilot clinical trial examining the effects of Aspart, randomly assigned for 12 weeks.	
INI Detemir	Study of Nasal Insulin to Fight Forgetfulness - Long-acting Insulin Detemir - 120 Days (SL120)	NCT01595646	11/11	03/17	Suzanne Craft, PhD-Wake Forest University Health Sciences	completed	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5409050/	This study was done to compare results with SNIFF-LONG 21	
INI Aspart	Memory and Insulin in Early Alzheimer's Disease (MAIN)	NCT00581867	10/07	05/12	Jeff Burns, MD-University of Kansas	completed	Study results not released.	Purpose to determine which parts of the brain are involved in insulin related memory improvement in AD and normal adults	

INI Glulisine	Intranasal Glulisine in Amnestic Mild Cognitive Impairment and Probable Mild Alzheimer's Disease	NCT02503501	08/15	09/18	Michael H Rosenbloom, MD-HealthPartners Institute	ongoing		Results include safety and effectiveness of Study of Intranasal Insulin Glulisine on Cognitive and Memory in Mild-Mod AD Patients	
INI Humulin	The Study of Nasal Insulin in the Fight Against Forgetfulness (SNIFF)	NCT01767909	01/14	12/18	Paul Aisen, MD (Univ. of Southern California) in collaboration with Suzanne Craft, PhD (Wake Forrest Univ.)	ongoing		Examining the effects of intranasally-administered insulin on cognition, entorhinal cortex and hippocampal atrophy, and CSF biomarkers in amnestic mild cognitive impairment (aMCI) or mild AD	
Metformin	Insulin Resistance and Mild Cognitive Impairment Study (IRMCI)	NCT02409238	04/15	12/17	Wee Kien Han Andrew, MCI-SingHealth Polyclinics	ongoing		Aim to reduce insulin resistance using exercise and weight loss + metformin treatment (Recruiting subjects with Prediabetes/ T2DM***)	Age: 55 or older, has mild cognitive impairment not dementia, no psychiatric disorder, no contradictions to metformin treatment, BMI greater than 23 or considered overweight based on country, no major health issues, no history of diabetes***
Metformin	Effect of Insulin Sensitizer Metformin on AD Biomarkers	NCT01965756	01/13	04/17	Steven E Arnold, MD- Upenn Memory Center	completed	Koenig et al. [222]	A Randomized Placebo Controlled Crossover Pilot Study of Metformin Effects on Cognitive, Physiological and Biochemical Biomarkers of MCI and Dementia Due to AD	
Metformin	Metformin in Amnestic Mild Cognitive Impairment	NCT00620191	02/08	02/12	Jose A Luchsinger, MD- Columbia University	completed	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5079271/	Compare brain function between metformin and placebo group using PET scan. ADAS-Cog also used for assessment.	

(*Continued*)

Table 1
(*Continued*)

Intervention	Title	ClinicalTrials.gov ID #	Start Date MM/YY	End Date MM/YY	PI(s) Sponsor(s)	Status	Reference(s)	Notes/Additional Information	Inclusion Criteria
Rosiglitazone	Insulin, Neurogenetics and Memory in Alzheimer's Disease	NCT00018382	10/99	03/03	Steven Kahn, MD -VA Office of Research and Development	completed	https://www.ncbi.nlm.nih.gov/pubmed/16286438	testing insulin sensitizing agent on patients with mild AD	Age 50–85, Mild AD/MCI without other serious medical or psychiatric conditions, no history of diabetes
Rosiglitazone	Brain Imaging Study Of Rosiglitazone Efficacy And Safety In Alzheimer's Disease	NCT00265148	04/04	07/08	GlaxoSmithKline	completed	https://www.ncbi.nlm.nih.gov/pubmed/16446752 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3214882/ https://www.ncbi.nlm.nih.gov/pubmed/20930300 https://www.ncbi.nlm.nih.gov/pubmed/21592048	Failed effects of rosiglitazone on improving functional brain activity and cognition. A series of similar studies also show failed results.	
Pioglitazone	Pioglitazone in Alzheimer Disease	NCT00982202	01/02	01/05	David Geldmaher, MD- NIA and Takeda Pharmaceutical Company	completed	https://www.ncbi.nlm.nih.gov/pubmed/20837824	Assessed the safety and tolerability of pio in non-diabetic AD patients	no psychiatric disorder, cardiovascular, or any other illness including diabetes
Pioglitazone	Biomarker Qualification for Risk of Mild Cognitive Impairment Due to Alzheimer's Disease and Safety and Efficacy Evaluation of Pioglitazone in Delaying Its Onset (TOMMORROW)	NCT01931566	08/13	07/19	Takeda Pharmaceutical Company and Zinfandel Pharmaceuticals	ongoing		Assessed the biomarker risk algorithm for prognosis of MCI or AD and the efficacy of pio in comparison to placebo	

Chromium	Effects of Chromium on Insulin Resistance in Alzheimer Disease Patients	NCT03038282	02/17	10/19	Andreana Haley, PhD- Metabolic Therapy Inc.	ongoing		Assessed the effect of chromium supplementation for AD individuals combined with exercise and the effect of supplementation on glucose metabolism.	diagnosed with AD and exhibits onset and progression of cognitive dysfunction 3 months prior to screening, no other disease or condition
NIC5-15	A Single Site, Randomized, Double-blind, Placebo Controlled Trial of NIC5-15 in Subjects With Alzheimer's Disease	NCT01928420	01/07	03/10	Hillel Grossman, MD-Humanetics Corporation, NCCIH, James J. Peters Veterans Affairs Medical Center	completed	Study results not released	NIC5-15 is a naturally occurring cyclic sugar alcohol acts as an insulin sensitizer that reduces AB production –	NINCDS/ADRDA criteria for probable AD, no history of diabetes, no other medical condition of disease
grape seed polyphenolic extract and resveratrol	BDPP Treatment for Mild Cognitive Impairment (MCI) and Prediabetes or Type 2 Diabetes Mellitus (T2DM) (BDPP)	NCT02502253	06/17	10/18	Sarah Lawrence, MS-Johns Hopkins University	ongoing		Assessing safety of BDPP, a nutraceutical, on humans after promising animal results in oral BDPP absorption.	Amnestic MCI, clinically stable diabetes, no dementia, ages 50–90

for 120 days to determine the benefits of a long acting insulin in comparison to short acting insulin for cognition, daily functioning, and AD biomarkers (ClinicalTrials.gov ID# NCT01595646). Preliminary results from this ongoing trial indicate that the regular insulin treated group had better memory compared to the placebo while no significant effects were measured for the detemir treated group [216]. In addition, the regular insulin treated group showed preserved brain volumes on MRI scans and reduced tau - P181/Aβ_{42} ratio. Another trial employing intranasal insulin aspart used evaluated brain regions involved in insulin-therapy related memory improvement using an fMRI measure of hippocampal activation (ClinicalTrials.gov ID# NCT00581867). Preliminary data reported greater hippocampal blood oxygenation level dependent (BOLD) activity in 3 AD subjects administered 40 IU insulin compared to 3 AD subjects administered saline. Additional cognitive tests show slight performance increases in subjects with the insulin condition although interpretation is hard due to the small sample size. This study is posted as complete yet no official results have been posted to CinicalTrials.gov.

In addition, there are ongoing clinical trials studying intranasal insulin delivery of the insulin analogs Glulisine (rapid-acting) and Humulin (human recombinant insulin) for their effectiveness in improving memory and functioning in comparison to placebo (ClinicalTrials.gov ID# NCT02503501, NCT01767909). These trials are scheduled for completion around the end of 2018. Currently, the clinical trial results released support the consensus for pursuing further studies for a longer treatment duration and larger sample populations. There seems to be a promising effect of insulin on memory improvement and cognition, but more study and validation is necessary.

Insulin sensitizer strategies

Like insulin, insulin sensitizers appear to impinge upon components of the insulin signaling axis and expression of genes that improve the neuropsychological profile of MCI and early AD [97]. Insulin sensitizers prescribed for diabetes include the TZDs RSG and PIO that target the nuclear receptor and transcription factor PPARγ, and the biguanide MFM that targets AMPK. Although previous large-scale clinical trials testing insulin sensitizers for AD failed to show efficacy, similar to the failure of many other AD drug candidates [217, 218], poor trial design such as inclusion of late-stage AD subjects and combined analysis with MCI, short treatment regimen, and missing biomarker assessment [217, 218]. In contrast, small trials on patients diagnosed as MCI/early AD have consistently shown that insulin sensitizers provide significant cognitive benefit [139–144].

Metformin

AMP-activated protein kinase is an important regulator of energy homeostasis and glucose metabolism. Among other negative consequences of aberrant Aβ production, it is well established that Aβ negatively affects the AMPK pathway which leads to mitochondrial deficiencies and eventually insulin resistance [219, 220]. The AMPK agonist MFM is highly effective in normalizing insulin resistance in type 2 diabetes [114, 115]. It attenuates AD-like neuropathology in mouse models of diabetes by reducing hippocampal tau phosphorylation and one of the tau kinases, c-jun N-terminal kinase [187], while also activating the tau phosphatase mTOR/protein phosphatase 2A in mouse primary neurons [188]. MFM also ameliorates AD-like molecular and neuropathological hallmarks [189, 221]. In addition, MFM reduces BACE1 with beneficial effects on Aβ production [190].

We found three clinical trials using MFM to treat subjects with AD-like cognitive impairment (Table 1). A SingHealth Polyclinic sponsored trial is currently recruiting patients that have both cognitive impairment and prediabetes and type 2 diabetes. It is important to note that the subjects have never been exposed to any kind of antidiabetic drug and will be exposed for the first time. The aim of this study is to test whether intensive lifestyle intervention with MFM treatment will increase cerebral glucose metabolism and cognitive function. By selectively choosing insulin resistant and diabetic subjects with cognitive impairment, this study will test whether MFM is both effective in treating diabetes while also improving cognitive function. This study is exclusive to 360 elderly Chinese adults with half of the subjects receiving MFM and lifestyle intervention and the other half with just lifestyle intervention for two years. This study is scheduled to end December 2017 (ClinicalTrials.gov ID# NCT02409238).

A completed interventional MFM trial sponsored by the University of Pennsylvania Memory Center aimed to study the effects of MFM on AD biomarkers in subjects diagnosed with mild cognitive disorder or early dementia due to AD with no history

of diabetes (ClinicalTrials.gov ID# NCT01965756). This was a randomized, double-blinded, placebo controlled study that enrolled 30 subjects to receive MFM for 8 weeks followed by placebo for another 8 weeks or vice versa. Cognitive biomarkers were measured using the Alzheimer's Disease Assessment Scale-Cognitive Sub scale (ADAS-COG) and Cambridge Neuropsychological Test Automated Battery assessments. CSF biomarker analysis and Arterial Spin Label MRI for changes in cerebral blood flow. The published report showed improved learning and memory after exposure to MFM but no significant changes in cerebral blood flow [222]. Given the small scope of this trial, further large-scale studies are warranted.

Another small, completed pilot MFM trial sponsored by Columbia University (ClinicalTrials.gov ID# NCT00620191) tested the use of MFM in subjects with amnestic MCI, overweight or obese, to determine the efficacy of MFM in prevention or slowing of conversion to AD. The study design was a 12 months double-blind placebo-controlled randomized pilot trial that separated 80 subjects into either a group that receives 1000 mg MFM twice daily or placebo. Study outcomes were measured using the Bushcke Selective Reminding Test (SRT), the ADAS-Cog, PET and MRI, and plasma Aβ levels. Significant results from this study include higher SRT values among subjects taking the highest MFM dose. ADAS-cog, PET, and MRI did not show statistically significant results with MFM [183]. Again, a larger trial was deemed warranted and designed to evaluate the efficacy and cognitive safety of MFM in prodromal AD.

Thiazolidinediones

Pharmacologically, members of the highly selective TZD drug class activate PPARγ; whereas endogenously, PPARs are activated by free fatty acids and the eicosanoids, derivatives of omega-3 and omega-6 fatty acids. Much is known regarding the role of PPARγ in peripheral tissues [223], its role in neuronal function emerged following immunohistological identification of PPARγ expression in brain areas associated with higher cognitive function, including the neurons of the cortex, basal ganglia, hypothalamus, and hippocampus [224–227]. PPARγ agonism is generally recognized as neuroprotective [228–231] via attenuated levels of pro-inflammatory proteins (e.g., iNOS, TNFα, MMP9), reactive oxygen species (ROS), and Aβ. Thus, PPARγ is a therapeutic target in many CNS diseases including Parkinson's disease [232–234], ischemia-reperfusion injury [235, 236], and traumatic brain injury [237, 238] in addition to AD.

We and others have demonstrated that PPARγ agonism improves cognitive performance in AD mouse models, predominantly in tasks that require intact hippocampal ERK signaling [72, 157, 239, 240]. Cognitive enhancement has been shown to be accompanied by improved AD biomarker profiles: alleviation of amyloid and tau pathology [239, 241–244], reduced neuroinflammation [244–246], increased antioxidant protection [246, 247], amelioration of central insulin resistance [104, 246], and normalization of several transcripts and proteins related to ERK and insulin signaling in the hippocampus, including reversal of downregulated PPARγ [153]. These findings reinforce the notion that modulators of ERK activity are promising therapeutic targets for early AD intervention.

In addition to alleviating cognitive dysfunction, TZD PPARγ agonism has been found to alleviate amyloid pathology through mechanisms involving enhanced Aβ clearance [243], suppressed expression of β-secretase and AβPP [248], as well as enhanced AβPP ubiquitination and subsequent degradation [249]. *In vitro*, TZD treatment reduced Aβ accumulation [250] and the expression of TNFα and interleukin-6 [251], factors that contribute to insulin resistance. Furthermore, application of Aβ to hippocampal slice cultures has been shown to inhibit Schaffer-collateral LTP, while TZD pre-treatment attenuated this effect and PPARγ antagonism reversed TZD effect [252]. Taken together, these data suggest a therapeutic role for PPARγ agonism to combat AD pathology in order to ameliorate associated network plasticity and cognitive deficits [154, 155, 253–257].

A dozen clinical studies have evaluated RSG in AD beginning in 1999 sponsored by VA Office of Research and Development (PIs: S. Kahn, S. Asthana, A. Fujimoto) followed by many additional trials performed by the developer of RSG, GlaxoSmithKline (Table 1). Unfortunately, all of these studies were designed and executed prior to the revised criteria for the staging and diagnosis of AD as recommended by the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for AD [258] and did not include currently accepted diagnostic, staging, and biomarker outcome measures.

Initially (ClinicalTrials.gov ID# NCT00018382), a 6-month trial administering 4 mg of RSG took

course as a treatment option for MCI or mild-AD subjects. Reports showed that there were improvements in attention and recall and stable plasma Aβ levels in the treatment group over a consistent decline in plasma Aβ in the placebo group. Thus, the results indicated that treatment group were slower in disease progression [139]. Several RSG clinical trials were completed by GlaxoSmithKline ranging from phase I safety to phase III efficacy studies beginning in 2004. These trials utilized ADAS-cog and CDR-SB as primary outcome measures under different RSG dosages and treatment regimens with no statistically significant effect on subjects with mild-to-moderate AD [140, 142, 259, 260]. Although some of the smaller trials using subgroup analyses indicated improved brain glucose utilization and APOE genotype effects, the failed large trials that followed prompted GSK to terminate and discontinue clinical trials evaluating RSG for AD.

A somewhat recent study completed by the National Institute of Aging and Takeda Pharmaceutical Company in 2005 tested PIO for efficacy and tolerability on non-diabetic AD subjects [261]. Results indicate that there were no adverse responses to PIO aside from peripheral edema after 18 months of treatment. Benefits for AD treatment were not indicated from this trial (ClinicalTrials.gov ID# NCT00982202). Currently, a 5-year clinical trial named "Tommorrow" sponsored by Takeda and Zinfandel Pharmaceuticals is underway. This trial is testing an algorithm that categorizes relative AD risk in test subjects based on ApoE and TOMM40 polymorphisms to assess PIO for delaying AD diagnosis. A total of 3,494 individuals with the desired genotype and normal cognition have been enrolled and assigned to a high risk or low risk group using the endophenotype algorithm. The high-risk group will receive PIO and the low risk group will receive placebo. Final data will be collected after the 5-year mark in July 2019 (ClinicalTrials.gov ID# NCT01931566).

Alternative approaches

Lifestyle and environmental factors are other active areas of study to ameliorate cognitive decline [262] and reduce pathology [263, 264]. Nonsteroidal anti-inflammatory drugs have been extensively tested in AD with mixed results [265] as have several natural compounds including ginko biloba [266], resveratrol [267], and cerebrolysin [268].

Here we discuss three insulin-related trials that targeted metabolic mechanisms in AD patients (Table 1). Metabolic Therapy Inc. tested the effects of chromium on AD patients to study whether chromium supplements combined with exercise will reduce insulin resistance and improve glucose metabolism. Chromium was used for its nutritional influence in "optimal insulin activity" and beneficial outcomes in other diseases like AD. The trial is still ongoing and scheduled for completion in October 2019 (ClinicalTrials.gov ID# NCT03038282). Another clinical trial by Humanetics Corporation uses NIC5-15 in treating AD. NIC5-15 is a naturally occurring sugar alcohol that has shown to be safe and effective insulin sensitizer through a smaller clinical trial performed prior to this trial. The study results were not reported, but the trial measured changes in cognition with ADAS-Cog after a combined treatment of NIC5-15 and a NMBA antagonist for 12 weeks (ClinicalTrials.gov ID# NCT01928420). The last clinical trial by Johns Hopkins University studied the effects of Bioactive Dietary Polyphenol Preparation (BDPP) on cognitive improvement in subjects with mild cognitive impairment with type 2 diabetes. BDPP is a combination of grape seed polyphenolic extract and resveratrol that was shown to improve cognition and memory in mouse models with AD and metabolic disorders. The study was divided into three treatment groups according to low, moderate, and high doses of BDPP administered orally. This trial is scheduled for completion in October 2018 (ClinicalTrials.gov ID# NCT02502253).

As is evident in the preceding paragraphs, clinical trial design varied in factors such as interventional strategy, duration, dose, and treatment group inclusion/exclusion criteria. Some clinical trials, such as the intranasal insulin trial with aspart, were specific in their aim to see the effects of the drug at different dosages. Other trials were more holistic and added other factors like diet and exercise to their interventional design. The wide range of interventional designs shines light to the idea that individual interventional methods were understood at different levels at the time. Thus, as more information is known about a treatment method through preliminary studies, it would be ideal to create more complex designs that address other risk factors instead of focusing on one factor. This is especially true since insulin and insulin-sensitizing agents in the brain are directly associated to metabolism and energy regulation in the periphery. Thus, incorporating healthy metabolic behavior may prove to complement

the effects of insulin and insulin sensitizing therapy.

SUMMARY AND CONCLUSIONS

Insulin signaling is a critical factor in brain homeostasis, metabolism, synaptic plasticity, and memory. Hippocampal memory failure is one of the earliest detectable traits of AD pathology [173, 269] with its high glucose needs and insulin sensitivity, it is not surprising that many clinical trials target the insulin signaling axis to address early AD cognitive impairment. This review has focused on our understanding of the molecular processes of hippocampal learning and memory as it relates to insulin resistance as a risk factor for AD and clinical trials that target insulin directly or common convergence points in the insulin signaling pathway as therapeutic intervention strategies. In support of the clinical trial strategies, we presented multiple lines of evidence from preclinical animal studies that cognitive deficits in AD models are triggered by Aβ-mediated neuroinflammation, insulin resistance, mitochondrial dysfunction, and impaired hippocampal ERK-dependent memory. We therefore postulated that progressive dysregulated hippocampal insulin/ERK signaling contributes to cognitive decline with clinical relevance for defining patient populations potentially responsive to mechanistically distinct insulin-sensitizer therapies. Thus, preclinical AD models can provide insight for disease stage-specific clinical trial design in humans with early AD.

We highlighted results from clinical trials that show promising effects of insulin and insulin-sensitizer therapy on treating currently accepted cognitive and biomarker outcome measures in appropriately defined patient populations. Of note is that each clinical trial held overlapping but often unique criteria for subject inclusion regarding history of diabetes, vascular disorders, psychological disorders, dementia rating, and AD diagnostic stage. While some patient populations may be excluded from trials based on the provided criteria, there is some gray area concerning treatment response on an individual basis. For instance, some of the trials target both MCI and mild-AD patients for treatment although MCI and AD are different forms of cognitive disorders [45, 47, 258] such that the same drug intervention may results in different outcome measures. Tailoring patient populations to treatment could be a more insightful way to run the trials.

A clinical trial led by Dr. Suzanne Craft may represent a first step toward addressing this issue (ClinicalTrials.gov ID# NCT03140865). In this study, defined patient populations are followed longitudinally to understand how each cohort digresses in condition without any interventional drugs. This study observes 5 different clinical populations over five years to identify early risk factors and characterize the progression of cognitive decline. The five groups are divided into individuals that have normal cognition with normal glycemic levels, normal cognition with prediabetes, mild cognitive impairment with normal glycemic levels, mild cognitive impairment with prediabetes, and AD. Once patient populations are understood in the pathology of their respective conditions, they could be tailored as ideal clinical trial subjects.

As presented and discussed here, insulin-centric clinical trials for AD and MCI subjects have clinically significant data that support larger, and longer interventional clinical studies as well as earlier disease-stage interventions to refine the setting for use of insulin sensitizers as AD treatment. New studies with genotypic (e.g., GWAS and risk loci) and phenotypic patient screening (e.g., glucose tolerance test for insulin resistance and FDG-PET imaging) focused on insulin-related mechanisms will be instrumental in breaking through with an interventional disease-modifying AD therapy.

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Mitochondrial Function, Dynamics, and Permeability Transition: A Complex Love Triangle as A Possible Target for the Treatment of Brain Aging and Alzheimer's Disease

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Abstract. Because of the failure of all amyloid-β directed treatment strategies for Alzheimer's disease (AD), the concept of mitochondrial dysfunction as a major pathomechanism of the cognitive decline in aging and AD has received substantial support. Accordingly, improving mitochondrial function as an alternative strategy for new drug development became of increasing interest and many different compounds have been identified which improve mitochondrial function in preclinical *in vitro* and *in vivo* experiments. However, very few if any have been investigated in clinical trials, representing a major drawback of the mitochondria directed drug development. To overcome these problems, we used a top-down approach by investigating several older antidementia drugs with clinical evidence of therapeutic efficacy. These include EGb761® (standardized ginkgo biloba extract), piracetam, and Dimebon. All improve experimentally many aspects of mitochondrial dysfunction including mitochondrial dynamics and also improve cognition and impaired neuronal plasticity, the functionally most relevant consequences of mitochondrial dysfunction. All partially inhibit opening events of the mitochondrial permeability transition pore (mPTP) which previously has mainly been discussed as a mechanism relevant for the induction of apoptosis. However, as more recent work suggests the mPTP as a master regulator of many mitochondrial functions, our data suggest the mPTP as a possible relevant drug target within the love triangle between mPTP regulation, mitochondrial dynamics, and mitochondrial function including regulation of neuronal plasticity. Drugs interfering with mPTP function will improve not only mitochondrial impairment in aging and AD but also will have beneficial effects on impaired neuronal plasticity, the pathomechanism which correlates best with functional deficits (cognition, behavior) in aging and AD.

Keywords: Antidementia drugs, inhibition of mitochondrial permeability transition pore function, mitochondrial dysfunction, therapeutic efficacy

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MITOCHONDRIAL DYSFUNCTION IN AGING AND DEMENTIA, A UNIFYING CONCEPT

Alzheimer's disease (AD) is characterized by neurodegeneration (synaptic deficits and finally neuronal loss) and the presence of histopathological alterations

(extracellular amyloid-containing plaques and intracellular tangles of hyperphosphorylated tau protein) as well as by severe cognitive deficits clinically often accompanied by neuropsychiatric symptoms. If or if not one or both of the two histopathological hallmarks play a causative role remains unclear for many decades. The discovery of homocygotic risk genes in most of the very rare (probably less than 1%) cases of early onset Alzheimer's disease (EOAD) which share increased production of amyloid-β (Aβ) as one (but probably not the only one) common property led to the hypothesis of Aβ as the major causative factor not only for EOAD but also for late onset AD (LOAD). These findings were supported by a large number of mainly preclinical data using transgenic cell and animal models finally leading to the amyloid cascade hypothesis [1] suggesting the slow accumulation of Aβ containing plaques as the major causative pathomechanism of AD, even if neurotoxic low molecular weight Aβ aggregates (oligomeric Aβ) were also seen to be relevant in the later years [2]. This hypothesis was strongly driven by the many transgenic animal models of AD which all show a substantial Aβ plaque load, although cognitive deficits and signs of neurodegeneration were often only remote at best and cognitive deficits did not correlate with Aβ levels [3–5]. Based on this hypothesis, many drug treatment strategies were developed to remove amyloid plaques (inhibitors of aggregation, inhibitors of the secretases producing Aβ from its precursor protein, antibodies to remove Aβ, or the increased production of antibodies by vaccination). Even if all seemed to remove Aβ to some extent, all strategies failed to improve the symptoms of dementia, some of the treatments made dementia even worse [6–9].

Accordingly, other aspects of AD pathology, more closely related to the clinical symptoms of the disease are currently investigated as targets for therapeutic improvement like the already mentioned synaptic deficits and impairment of synaptic plasticity [10–13]. Synaptic plasticity, the dynamic regulation of synaptic mechanisms like LTP (long-term potentiation), spine density and form, number and length of dendrites and axons (neuritogenesis), and the number of neurons (neurogenesis and apoptosis) represents a major mechanism by which our brain can adapt to periods of pathologically enhanced or reduced function or to save information at the synaptic level. Mitochondria play an important role as they provide the cellular energy (ATP) for these adaptive responses or initiate apoptosis in case of neuronal damage beyond the possibility of repair [14–16]. Changes of synaptic function and plasticity play a major role for cognitive deficits in aging and dementia [2, 5, 13, 17–20]. Synaptic deficits always showed the best correlations with clinical symptoms of AD patients already in one of the first studies published [21] and also correlated with functional impairment in AD mouse models [5].

Detectable long before the clinical manifestation of AD, impaired cerebral glucose metabolism in several brain regions, most likely due to impaired mitochondrial function, represents a very early pathomechanism of AD [22]. This parallels many other observations of mitochondrial deficits in AD brains like reduced activities of mitochondrial enzymes and of complexes of the respiratory chain and increased oxidative stress due to elevated free radical (ROS) damage [23]. Mitochondrial dysfunction is also a common feature of all AD mouse models [3, 23–25]. Mitochondria are abundant in synaptic terminals since ATP production by mitochondria is crucial for synaptic function. Consequently, impaired mitochondrial function associated with reduced ATP supply leads to synaptic dysfunction, reduced neuronal and synaptic outgrowth, and finally apoptosis [8, 14, 23]. Drugs which improve mitochondrial function enhance neuronal survival and improve neurite outgrowth and neuronal proliferation [27–30].

Both histopathological alternations of early and late onset AD (EOAD and LOAD) like elevated Aβ levels as well as the presence of neurofibrillary tangles and most other relevant risk factors like brain aging, microvascular dysfunction, APOE4 genotype, mtDNA polymorphisms, and gender converge at the level of impaired mitochondrial function [3, 24, 25, 31]. As synaptic function and synaptic plasticity strongly depend on energy (ATP) mainly provided by the mitochondria, mitochondrial dysfunction is closely associated with synaptic deficits in aging and AD [14, 26, 32–36]. These observations led to the hypothesis that impaired mitochondrial function, associated with reduced energy metabolism and enhanced oxidative stress as well as synaptic dysfunction represents a common final pathway of all specific (genetic) and non-specific risk factors for the development of AD [23, 25, 37]. This concept has been put forward in the "mitochondrial cascade hypothesis" first proposed more than 10 years ago by Swerdlow and coworkers [8, 38, 39]. This concept suggests mitochondrial dysfunction not only as the major pathomechanism of AD which slowly develops by aging but also as major driving force for the slow decline from aging to AD. Initially caused by

the combined effect of oxidative stress due to aging and slightly elevated Aβ levels caused by individual risk factors, mitochondrial impairment starts to develop long before Aβ deposits begin to form. Further driven by genetic, environmental, and individual factors, mitochondrial dysfunction associated with elevated free radical (ROS) production cumulates in susceptible patients over many years. This process is self-accelerating as ROS will further damage mitochondria which respond with further elevation of ROS. At some point, elevated ROS production will reach a level where Aβ production increases due to β-secretase and γ-secretase activation [37, 40]. Aβ in turn will further impair mitochondrial function and will aggregate to fibrils and finally to plaques. This scenario suggests that Aβ still has a causative role but it is not necessarily the major player. It also seems to be a side product once aggregated to plaques without major functional relevance. This could easily explain that Aβ deposits themselves do not correlate with early signs of neurodegeneration or impaired cognition [41, 42].

The major aspect of this concept relates to mitochondrial dysfunction as the major pathomechanism directly driving neurodegeneration and psychopathology independently of Aβ deposits, from the initial phase of the disease, long before a clinical diagnosis becomes possible, to the later phases of mild to moderate dementia. Accordingly, mitochondrial dysfunction can lead to early signs of neurodegeneration or synaptic deficits as well as distinct cognitive deficits without Aβ deposits being present [43–47]. Additional proof for the mitochondrial cascade hypothesis of dementia may come from studies with mitochondria targeted drugs which should be able to improve cognitive impairment over the whole aging spectrum.

PHARMACOLOGICAL STRATEGIES TO IMPROVE MITOCHONDRIAL FUNCTION

While the concept of mitochondrial dysfunction as a major pathomechanism for the cognitive decline in aging and AD has received substantial support over the last decade, improving mitochondrial function as a strategy for new drug development has not. Preclinical data about improvement of mitochondrial dysfunction and associated deficits of synaptic function and neuronal plasticity as well as cognitive deficits have been reported for several antioxidants, for many polyphenols and other natural compounds, and for some newly developed synthetic drugs. This research was mainly driven by the concept to identify possible targets and/or to investigate effects on individual aspects of mitochondrial function. These studies addressed many aspects of the mitochondrial machinery and investigated known or newly developed compounds [25, 48–53]. For only few of the investigated compounds effects with possible clinical relevance have been reports in animal models, very few if any have been investigated in clinical trials. The lack of clinical evidence or even proof represents a major drawback of the mitochondrial directed drug development. With the limited data available it appears that radical scavenging activity alone (vitamins C and E) is not sufficient for clinical improvement [49]. Compounds which show some clinical benefit seem to act directly at the mitochondrial level [49, 50] and improve one or more mechanisms of impaired mitochondrial function (ATP production, Oxphos activity, synaptic plasticity, mitochondrial dynamics, mitophagy) but a clear common final target mechanism has not yet been identified. A typical example is curcumin which improves many aspects of mitochondrial function *in vitro* but shows mixed results in men probably due to the low bioavailability of the preparations used so far [49, 50].

To overcome these problems, we used a different "top-down" approach by investigating several older antidementia drugs with clinical evidence of therapeutic efficacy in aging and dementia although not always in line with our todays diagnostic standards for clinical studies. These include EGb761® (standardized ginkgo biloba extract), piracetam, and Dimebon [30, 54–56]. All of them improve specific aspects of mitochondrial function and mechanisms of mitochondrial quality control relevant for mitochondrial dysfunction as present in aging and dementia. Moreover, they seem to affect the mitochondrial permeability transition pore (mPTP) as common target.

Piracetam and ginkgo extract have a long history as so-called "nootropic drugs" which improve cognitive functions in a variety of conditions related to elevated oxidative stress according to our previous concepts including AD and vascular dementia (VaD), aging, and brain injuries) [30, 57]. Pharmacologically both drugs improve energy production (ATP) and glucose metabolism leading to the alternative term "metabolic enhancer" [30, 54, 56]. Even if both drugs showed efficacy in early clinical trial using the dementia concepts of those times, both were seen subsequently rather critically because of the lack of a disease

related mechanism of action and of limited therapeutic efficacy. However, when it became known that oxidative stress and impaired mitochondrial function might be a common pathomechanism underlying the various conditions of cognitive deficits mentioned above, the pharmacology of both drugs needed to be reconsidered. Moreover, recent comparisons suggest that clinical efficacy is rather comparable to the acetylcholinesterase inhibitors as the standard treatments for AD [30]. Moreover, with increasing knowledge that all "disease-modifying" Aβ directed AD therapeutic concepts failed, the acceptance of a non-specific mitochondria directed treatment concept increased substantially in the last years.

Clinical efficacy in aging and dementia

EGb 761® has been widely used since its introduction into the market to improve deficits of cognition over a large range of conditions from aging to dementia. However, scientific proof for its use was always seen very critically because of the large range of cognitive disturbances investigated (it is not yet long ago that mild cognitive dysfunction in aging was considered to be completely different from mild stages of AD) and the different and sometimes poor design of some of the older studies. However, when adequate methods were used for the individual conditions, EGb761® sets an example that a mitochondrial-directed drug not only shows substantial clinical benefit in AD but also shows clinical efficacy in patients with mild age-related cognitive deficits and in patients with VaD [30, 56]. This concept is completely different from the concept for drugs related to the amyloid hypothesis which assumes that AD drugs must work via Aβ and therefore cannot be efficacious in VaD. Thus, the broad preclinical and clinical activity of EGb761® might be representative for all future drugs improving mitochondrial dysfunction and cognitive impairment over the whole spectrum of age-related memory disorders [30].

Another example is the metabolic enhancer piracetam, the prototype of the so-called "nootropic" drugs [58]. Piracetam has been shown to improve impaired cognitive functions in various conditions in men from aging, dementia, and brain injuries [54, 57, 59]. Even if its clinical usefulness is seen controversially, piracetam is still used in many countries to treat cognitive impairment in aging and dementia, following brain injuries and stroke, as well as after coronary surgery. A meta-analysis of all available (published and not published) clinical studies provided substantial evidence for a global efficacy in a diverse group of older subjects with cognitive impairment [57]. As it was the case for ginkgo, this broad efficacy was seen very skeptically, but in our days appears to be typical for a mitochondria targeted drug. Contrary to EGb761®, recent clinical data in AD patients are not available. Older placebo-controlled double-blind studies where substantial improvement was seen also used clinical dementia concepts which included patients with AD and VaD [60, 61]. However as outlined above, this drawback gets less relevant in view of our recent concepts of mitochondria targeted drugs as typically seen in case of EGb761® where similar clinical efficacy has been reported for AD and VaD [30, 56]. For both drugs, there is a large range of clinical response (good, moderate, no response) explaining that the clinical data also include negative studies as discussed in the meta-analyses [30, 54–57, 61]. It will be a major challenge for the future to identify conditions for good clinical response like the presence of neuropsychiatric symptoms in the case of EGb 761® [30] and probably for Dimebon (see below).

Dimebon (latrepirdine) represents an old antihistaminic drug (first generation H1-antagonist) originally developed and clinically used in Russia as an anti-allergic drug [62]. Based on some preclinical studies including improvement of mitochondrial function and findings about robust cognition enhancing properties in a small group of AD patients, a large placebo controlled phase II trial was carried out in nearly 200 AD patients indicating substantial therapeutic benefit over placebo after 24 weeks not only for cognitive symptoms and for activities of daily living but also for neuropsychiatric (mainly affective) symptoms [64]. Dimebon's large effect was also driven by an improvement over baseline and much more by a reduction of the typical deterioration of AD symptoms as shown in the placebo group. The substantial therapeutic effects of Dimebon remained stable in a continuation phase over additional 6 months. However, a larger consecutive trial in AD patients failed to show positive effects of Dimebon over a similar study time (6 month) and for a similar Dimebon dose (20 mg tid) [65]. Contrary to the initial trial [64] where the placebo group got worse over 6 months (a reduction on the ADAS-cog scale by about 2.0 points), the placebo group in the second trial improved over 6 months by 1.2 ADAS-cog points [66]. However, because of major differences of design and patient characteristics of both clinical studies, because of the rather atypical patients selected for

the second trial (no deterioration over time), and regarding the extensive data about effects of Dimebon at the mitochondrial level as reviewed next, it appears that clinical efficacy was mainly associated with a slowing down of the progression of the disease [55, 66]. Quite interestingly, deterioration over time seems to be much more pronounced in patients with high levels of neuropsychiatric symptoms [30, 55]. This pattern seems to be typical for mitochondria targeted drugs as discussed recently for ginkgo extract [30]. Quite interestingly, the patients of the second trial had rather low levels of neuropsychiatric symptoms which, however, also improved with Dimebon treatment [66].

Effects on cognition

EGb 761® is a special dry extract of ginkgo leaves made with acetone 60% (w/w) as extraction solvent developed by the companies Schwabe (Germany) and Ipsen (France) more than 40 years ago. Relative to the original composition of the leaves, pharmacologically active components (flavonoids, terpene lactones) are enriched and possibly toxic components (ginkgolic acids) are downgraded. Nearly all of the clinical studies and the majority of preclinical studies published for ginkgo over the last decades used this standardized extract [30, 56, 67, 68]. After early studies with EGb 761® in patients with cerebral vascular disease reported positive effects on cognition [67], many experimental investigations in mice or rats confirmed improvement of cognitive functions (see the summary about older studies by Müller and Chatterjee [68]). These effects include improvements in many different cognitive domains like learning, short term memory, and aspects of working memory. Most of these older studies already reported better effects on cognitive performance in aged than in young or adult animals [68]. These initial observations have been confirmed in many subsequent studies, extending the better improvement of cognition from aging to overexpression of human Aβ (AD mice), to hypoxia, and cerebral vascular impairment, situations typical for the aging continuum of the mitochondrial cascade hypothesis [30, 56]. With respect to the mitochondrial cascade hypothesis of dementia, it is important to note that EGb 761® also improves mitochondrial dysfunction in aging or other situations of impaired brain function [24, 50, 70, 80].

Piracetam also improves impaired cognitive functions in various experimental conditions in men and in many animal models of impaired brain function as it seems to be typical for mitochondria targeted drugs reviewed in the present communication [54, 58, 71, 73, 74]. Similar to ginkgo, improvement was usually only seen when cognition was impaired by conditions associated with increased oxidative stress. Young animals or humans usually do not benefit from piracetam treatment.

Because of the complete failure to show any pro-cognitive effect in the second AD trial as reported above, it is important to review several animal studies reporting improved cognition after Dimebon administration. Giorgetti et al. [72] reported improved object recognition behavior at single oral doses leading to brain concentrations between 1.7 and 170 nmol/L, where maximal effect was already seen at 5 nmol/l. Cognition improving effects were also seen after 31 days of treatment in a transgenic mouse model expressing high Aβ levels but not in the non-transgenic littermates [75]. Dimebon also enhanced cognition in rats after lesions of the cholinergic forebrain system [76]. Improved cognition in a hippocampus-dependent learning task was also found in mice after acute or repeated dosing with Dimebon [57]. Similarly, Dimebon improved working memory in adult and aged monkeys at rather low doses and also in adult animals after impairment with scopolamine [78].

In a mouse model for depression, aged but not young animals showed anhedonic like behavior (reduction of sucrose preference) [79]. In possible analogy to the beneficial effects of Dimebon on neuropsychiatric symptoms in both AD trials [3, 6], treatment of aged (18 months) but not of the young (3 months) mice with Dimebon for 4 weeks reduced the anhedonic profile [79]. Plasma levels measured in some of the studies correlated quite well with plasma levels seen in AD patients [64, 66].

Effects on mitochondrial function

EGb761®

EGb 761® directly scavenges free oxygen species (ROS) as it can be expected from its flavonoid fraction [81]. This property is not shared by piracetam and Dimebon. Moreover, many experimental studies have clearly shown that EGb 761® additionally reduces mitochondrial ROS production and protects mitochondria and the complexes of the mitochondrial respiratory chain from further damage by ROS, improves the reduced mitochondrial membrane potential, and enhances glucose metabolism and the availability of ATP. Bilobalide and the

different ginkgolides are important for these properties [80, 82, 83]. As consequence, neuronal function improves especially following previous impairment (aging, hypoxia, hypoglycemia, elevated Aβ, cerebrovascular pathology) [80, 84–86]. Positive effects of EGb 761® on neuroinflammation may also be secondary to its effects on mitochondrial function [87, 88]. In line with these positive effects of EGb 761® on mitochondrial function outlined above, EGb 761® improves synaptic function and plasticity in a large number of cell and animal models [69]. Its effect is usually mainly seen when these parameters are impaired due to experimental conditions of reduced energy supply by aging, Aβ overexpression, hypoglycemia, or hypoxia, all having in common enhanced oxidative stress and impaired mitochondrial function. All aspects of synaptic plasticity have been shown to benefit from EGb 761® treatment including neuritogenesis, spine density, LTP, and neurogenesis [69, 89, 90].

Piracetam

As a very sensitive indicator for improvement of mitochondrial function, piracetam increased MMP in various cell models *in vitro* after impairment following many conditions related to aging, oxidative stress, hypoxia, Aβ exposure and also in animal models for brain aging and dementia *in vivo* and *ex vivo* [71, 73, 74]. This is parallel with observations of enhanced glucose metabolism and ATP production by piracetam [73, 74]. Ours and others studies indicate substantial neurotrophic properties of piracetam following impairment by oxidative stress like neuritogenesis [28, 73, 74] and neurogenesis [91, 92]. Initial findings suggested that these effects might also be associated with effects on mitochondrial dynamics [93]. In a subsequent communication, we confirmed effects of piracetam on neuritogenesis in a human cell model of LOAD [94] related to the mitochondrial fission and fusion balance (dynamics) and the inhibition of the mPTP opening [28]. Similar findings were obtained for the piracetam analogue levetiracetam [27].

Dimebon

Similar to ginkgo and piracetam, Dimebon also shows substantial positive effects on impaired mitochondrial function [55, 95, 96]. After treating mouse primary neurons or SY5Y neuroblastoma cells with Dimebon at low concentrations (1–10 nmol/l), enhanced mitochondrial membrane potential and ATP production can be measured. Under stress situations (elevated intracellular calcium, serum depravation), Dimebon also protected the cells against the decrease of mitochondrial membrane potential and led to better survival (reduced apoptosis) [97]. Impaired glucose utilization associated with aging has not only been demonstrated in human brains but also in the cortex, hippocampus, and somewhat less the cerebellum of mice [98]. Treatment of aged (20 months) but not of young (3 months) mice with Dimebon 75 min before measuring of glucose uptake with the PET tracer 18-fluoro-deoxyglucose showed significantly enhanced glucose uptake as indicator for a restoration of impaired glucose metabolism [98]. These findings fit nicely into our findings about effects of Dimebon on oxidative phosphorylation activity in HEK cells [99, 100]. Treating HEK control cells with 100 nmol/l Dimebon had no effects on OXPHOS activity as measured by high resolution respirometry. The same treatment significantly enhanced OXPHOS activity in HEK cells where OXPHOS was reduced by the overexpression of Aβ or by rotenone treatment as a model for the impairment of complex I function during aging [99, 100].

Similarly to ginkgo and piracetam, mitochondrial improvement by Dimebon (up to 100 nmol/) has been associated with enhanced neurite outgrowth in several cell systems [101–103]. Dimebon also enhances neuronal cell proliferation and neurogenesis [75, 104].

Improvement of mitochondrial quality control

Mitochondrial dysfunction as it occurs in aging and many neurodegenerative diseases like AD usually takes years or even decades before symptoms arise, since it only gets functionally relevant when the rate of damage exceeds the rate of continual repair by the mitochondrial quality control system. Mitochondrial dynamics, meaning the ability of mitochondria to undergo changes in size and form [105], are gaining more and more attention as an important factor regulating mitochondrial function and as a mechanism of mitochondrial quality control and seems to be substantially impaired in AD [106, 107]. Even if reports are sometimes controversial, in most cases mitochondrial fragmentation is accompanied by reduced mitochondrial function and vice versa [108–110]. Accordingly, shorter mitochondria seem to be energetically unfavorable. We have previously used confocal microscopy of fixed mitochondria as a very reliable method to analyze mitochondrial

dynamics in many situations of impaired mitochondrial function, where as a common feature the fission and fusion balance is shifted to the energetically less favorable fission site [24, 27, 28, 99].

We initially used HEK cells overexpressing Aβ with a pronounced shift of fission and fusion balance to the smaller size mitochondria. Treating these cells shifted back fission and fusion balance to the fusion site as shown for Dimebon [99], piracetam [28, 93], and ginkgo (Müller et al., unpublished findings). Similar effects have also been reported for the ginkgo ingredient ginkgolide K [111]. We also used human SY5Y cells slightly overexpressing Aβ as a model for LOAD and were able to confirm this effect for piracetam and levetiracetam [27, 28].

Mitochondrial fission is regulated by the interaction of mainly two proteins: the cytosolic GTPase dynamin-related protein 1 (Drp1) and an outer mitochondrial membrane anchored protein, mitochondrial fission protein 1 (Fis1). Fusion processes are chiefly regulated by the two GTP-ase isoforms: mitofusin 1 and 2 (Mfn1 and Mfn2), as well as optic atrophy type 1 (OPA1) protein. Parallel to the effect on mitochondrial dynamics, Dimebon and ginkgo reduced the elevated levels of the fission protein Drp1 [55, 111].

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE AS COMMON TARGET

The mPTP represents a dynamic multiprotein complex, which spans the inner and outer mitochondrial membranes at special contact sites. Although, the structure of the mPTP is not yet fully elucidated, there are several identified components or modulators of the mPTP. The most common proposed structure of mPTP includes the voltage-dependent anion channel (VDAC) and the 18 kDa translocator protein (formerly known as the peripheral benzodiazepine receptor) in the outer membrane, the adenine nucleotide translocator (ANT) in the inner membrane, cyclophilin D from the matrix, and possibly other proteins such as creatine kinase from the intermembrane space, and hexokinase at the outer surface of the outer membrane. Opening of mPTP plays a causative role not only in apoptosis by releasing cytochrome c but also in mitochondrial fragmentation. Inhibition of mPTP showed both reduction in expression of fission proteins and increase in expression of fusion proteins and an impaired fission and fusion balance [27, 28, 113, 114].

Numerous effectors can open the mPTP, in particular calcium ions, ROS, Aβ, and atractyloside as experimental compounds. On the other hand, many endogenous and exogenous inhibitors of mPTP have been described including high negative potential, low matrix pH, ADP, magnesium and strontium, and the immunosuppressive drug cyclosporine A, which was used in our experiments as a control where it inhibited mPTP opening induced by calcium ions [27, 28, 99]. Induction of mPTP leads to a nonspecific high permeability for different agents, to a collapse of MMP and loss of ATP. Mitochondria become permeable to all solutes up to a molecular mass of about 1500 Da and undergo a dramatic swelling. This finally ends in the rupture of the OMM and release of proapoptotic intermembrane proteins into the cytosol like cytochrome c [117, 118]. Cyclosporine A inhibits mPTP trough interaction with cyclophilin D [117]. Similar to cyclosporine A, all three antidementia drugs investigated function as inhibitors of mPTP opening by different agents like calcium, atrytyloside, and oxidative stress as reported for Dimebon [99, 100, 119, 120], piracetam [28, 121], and ginkgo extract as well as some of its ingredients [111, 122, 123].

FINAL CONCLUSIONS AND OUTLOOK

Even though there are still multiple models and viewpoints regarding mPTP and its components, the prevention of mPTP opening has been shown to provide neuroprotection in different paradigms by inhibiting the induction of apoptosis [115, 116, 124, 125]. However, more and more data suggest that beside its role in the regulation of apoptosis, the mPTP functions as a master regulator of all mitochondrial functions including OXPHOS activity, ATP production, MMP, and dynamics which are typically affected in aging and dementia and which benefit from the three antidementia drugs investigated (Fig. 1). Moreover, reducing mPTP function by reducing the concentration of one of its individual components improves mitochondrial function, synaptic deficits, and cognition as shown for cyclophilin D deficiency [132] and reduced VDAC1 levels [133]. Vice versa, elevating cyclophilin D levels impairs mitochondrial function, synaptic plasticity, and cognitive performance [137]. Thus, it appears quite plausible that interfering with mPTP

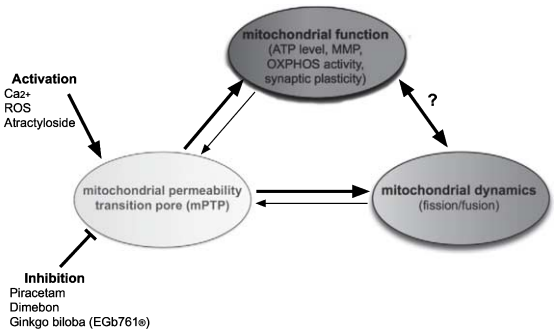

Fig. 1. The love triangle of mitochondrial function, dynamics, and mPTP function. Beside its well-known function as regulator of programmed cell death, there is increasing knowledge that the mPTP works as a master regulator of different mitochondrial mechanisms including MMP and ATP production and mitochondrial quality control, especially fission and fusion balance [28, 113, 114, 135]. Inhibition of PTP function has been shown to improve many aspects of mitochondrial function and impaired synaptic plasticity and to shift fission and fusion balance to the fusion side. For example, in a cell model of LOAD, piracetam shifted back mitochondrial fission and fusion balance to larger mitochondria accompanied by reduced mPTP opening events and improved neuritogenesis [28]. Moreover, mPTP opening by atractyloside was accompanied by enhanced fission which also was reduced by piracetam [28]. Consequentially, altering mitochondrial dynamics by down-regulation OPA1 was leading to larger mitochondria and reduced mPTP function [135]. Similarly, enhancing mitochondrial fusion by upregulating Mfs2 reduced the sensitivity of mPTP to opening by ROS [136]. Moreover, impairing mPTP function by downregulating individual mPTP compounds improves mitochondrial function synaptic deficits, and cognition as shown for cyclophilin D 132] and VDAC1 [133]. Vice versa, elevating cyclophylin D levels impair mitochondrial function, synaptic plasticity, and cognitive performance [134].

opening events represents a major mechanism by which the three antidementia drugs improve disturbed mitochondrial function and finally enhance neuronal plasticity. The mPTP has already been suggested by several authors as promising target to treat age-related neurodegenerative disorders [116, 126, 127, 130]. However, up to now, a relationship between inhibition of mPTP opening and therapeutic improvement in age-related memory impairment was missing. Our data summarized in the present communication can fill this gap and span a bridge from mitochondrial improvement to therapeutic outcome in dementia and might be very important for the future development of mitochondria targeted antidementia drugs. On the other hand, one should be quite careful in over-interpreting this concept as the therapeutic benefit for the three drugs is limited as summarized above. Even for EGb761®, which has the best data according to today's standards, effect sizes and percentages of patients responding to treatment are modest and are within the range reported for acetylcholinesterase inhibitors as our present standard treatment of AD [30, 128, 129]. On the other hand, this moderate response is obtained without major side effects for of all three drugs.

This will give rise to several important questions:

1) Up to now, therapeutic benefit for mitochondria targeted drugs is modest [30, 54, 55, 139]. Is this already the best possible or do we have the chance to develop mitochondria targeted drugs/drug combinations with better efficacy? Based on the data available we think that other mitochondria targeted drugs can show better efficacy but it seems rather unlikely that a magic bullet will be found. This has to be seen against the background of the many disappointments in the field and the perspective that no other approach to treat age-related memory disorders can be expected for the time coming.
2) Which models to test mitochondrial function should we use as there is some discrepancy between the sometimes substantial mitochondria improving effects of the three drugs in preclinical settings and the modest clinical efficacy? Adequate animal models seem to be mandatory.
3) The three drugs described in the present communication are quite different. All three only show modest impairment of mPTP function but not complete inhibition. How much inhibition is possible without blocking beneficial effects of mPTP opening like regulating programmed cell death [141]?
4) The mPTP represents a very complex system. Is it suitable as a specific molecular drug target? Which of the individual components should be targeted [139]? The three drugs described in the present communication are chemically and pharmacologically quite different and very likely interfere with different parts of this large supramelucular structure.
5) Should we continue to develop mitochondria targeted drugs or should we still wait for the right Aβ-directed drug even if all developments investigated so far failed to show relevant clinical benefit? It appears very unlikely that any other compound following this line will be better. Moreover, there is nothing on the horizon giving hope for the magic bullet within the next decade. Thus, it seems plausible to follow the mitochondrial concept which could result at least in reasonable efficacious drugs in a not too long time.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9915).

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Perspectives on Oxidative Stress in Alzheimer's Disease and Predictions of Future Research Emphases

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Abstract. Oxidative stress, an overproduction of free radicals or a diminution of free radical scavenging ability relative to those of cognitively aged-matched controls, is widely recognized as a critical component of the pathogenesis and progression of Alzheimer's disease (AD). This recognition arose in significant part from the work in the author's laboratory, complemented by research from others' laboratories. The Butterfield laboratory discovered the oxidative stress associated with oligomeric amyloid-β peptide manifested primarily as elevated oxidative modification of proteins and peroxidation of lipids. Such oxidative damage caused neuronal death, which undoubtedly underlies the progressive loss of cognition in AD. Identification of specific oxidatively modified brain proteins in subjects with AD or amnestic mild cognitive impairment was achieved by the methods of redox proteomics, pioneered in the author's laboratory. The importance and significance of the research emanating from the Butterfield laboratory rest on the paradigm shift of thinking regarding the roles of oxidative stress and resulting damage to key proteins and biochemical pathways in the pathogenesis and progression of AD. Predictions of future research directions also are presented. Given the enormous financial and personal burden placed upon citizens (and governments) of the US from AD, and the surety that the number of AD patients will greatly increase over the next 20–30 years, greater understanding of the molecular basis of pathogenesis and progression of AD is essential. Our laboratory is privileged to have contributed to better understanding of AD and provided rationales to identify effective therapeutic targets for this devastating dementing disorder.

Keywords: Alzheimer's disease, lipid peroxidation, neuronal death, oxidative stress, pathogenesis, predictions, progression, protein oxidation, redox proteomics

PERSPECTIVES ON THE IMPLICATIONS AND IMPORTANCE OF ALZHEIMER'S DISEASE RESEARCH PUBLISHED FROM OUR LABORATORY

The Butterfield laboratory is probably best known in the field of Alzheimer's disease (AD) research for two major discoveries: 1) The role of oxidative and nitrosative stress in brain and resulting paradigm shift in thinking about in the pathogenesis and progression of AD [1–5]; and 2) The pioneering of redox proteomics methods with which identification of oxidatively and nitrosatively modified, and consequently dysfunctional, brain proteins was achieved in specimens from subjects with AD and amnestic mild cognitive impairment (MCI) and the resulting new insights gained into key molecular pathways affected in these disorders [6–9].

Oxidative and nitrosative stress

Oxidative stress results when the production of oxygen-containing free radicals or molecules from which free radicals could be formed exceeds the

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rate of scavenging of such moieties by antioxidant enzymes or endogenous small molecule antioxidants [10, 11]. Oxidative stress is indexed in protein oxidation by formation of protein carbonyls and in lipid peroxidation, by among other molecules, 4-hydroxynonenal (HNE) [8, 10, 11]. By Michael addition, a covalent bond is formed when HNE binds to Lys, His, and Cys amino acids in proteins, changing their conformation and function [12]. Nitrosative stress involves actions of oxygen-containing moieties that also contain nitrogen. The most important nitrogen source in this context is nitric oxide (NO), a free radical formed from the action of nitric oxide synthase. Because radical-radical recombination reactions are among the fastest known chemical reactions, NO reacts with incredibly fast kinetics with superoxide free radical anion to form peroxynitrite ($ONOO^-$), a non-radical moiety [13]. This agent in the presence of carbon dioxide leads to nitrogen dioxide (NO_2) [13, 14], a free radical that, because the -OH group on an aromatic ring is ortho/para-directing, binds to the 3-position of tyrosine to form 3-nitrotyrosine (3-NT) [10, 11, 13, 14]. Protein carbonyl- and 3-NT covalent modifications on proteins are formal oxidations from a chemical point-of-view. Therefore, from henceforward in this paper, I will use the term "protein oxidation" in discussing both of these covalent modifications of proteins. As noted above, HNE formation is a reactive product of and marker for lipid peroxidation, that when covalently bound to proteins changes their structure and modifies their function [5, 8, 10–12, 15].

Oxidative stress in AD and MCI brain

Prior to the discovery from our laboratory and that of others that amyloid-β peptide (Aβ) oligomers led to oxidative modification of neuronal proteins and lipids [16–25], it was difficult to rationalize why there were so many reports of altered proteins, enzymes, lipids, and biochemical pathways in AD brain. Why was it not the case that only one major change occurs in AD brain that could account for the progressive pathology of AD and increasing cognitive decline throughout the stages of this dementing disorder?

The discovery of $A\beta_{42}$-mediated oxidative damage in neuronal cultures, synaptosomes, and in brains from both animal models of AD and subjects with AD and MCI [5, 19–33] opened the possibility that damage following oxidative stress could help explain the following observation: wherever in the brain $A\beta_{42}$ was abundant, oxidative stress occurred, and in contrast, wherever $A\beta_{42}$ levels were absent or low (i.e., cerebellum), excess oxidative stress in AD and MCI over that of aged-matched control cerebellum did not occur [28]. Different proteins and lipids, modified by the actions of $A\beta_{42}$ or from free radicals from other sources, have decreased function [34, 35]. Such a notion in AD, first proffered from the Butterfield laboratory, was a paradigm shift in thinking about the pathogenesis and progression of AD; that is, oxidative stress is a key factor in the pathogenesis and progression of AD [36]. This "radical" idea (please pardon the pun) is now generally accepted dogma about the pathogenesis and progression of AD [37–45].

However, a troubling question about the importance of oxidative stress in AD is: since protein oxidation and lipid peroxidation decreased protein activity after covalent modification or led to their decreased abundance, why have clinical trials employing antioxidants been such failures in AD [46, 47]? Several reasons may address this question. 1) Such clinical trials often occurred late in the disease when neuronal loss is rampant and therefore AD would not be susceptible to potentially positive interventions. Given that AD neuropathology is thought to occur approximately 20 years prior to the onset of clinical symptoms of this disorder [48], the ideal time for antioxidant therapy likely would be at the start or prior to initiation of AD neuropathology. Such a time frame would require some reliable set of biomarkers unique to AD in order to know who should be treated. This notion is discussed further below in the Future Research Directions section of this current paper. 2) Antioxidants in many clinical trials may not have been applied in the most effective manner or had poor penetration of the blood-brain barrier (BBB). For example, vitamin E, which traverses the BBB via a specific transporter [49], requires vitamin C or other agent to reduce the oxidized vitamin E back to vitamin E, i.e., so vitamin E can act as a continuous scavenger of free radicals and not as a saturable sponge-like molecule. This approach often was not the case in clinical trials of antioxidants in AD, likely due in part with the underappreciation of free radical chemistry. 3) The cellular redox state of individuals involved in the clinical trials usually was not considered. Accordingly, a person with a more reductive cellular redox state would not benefit from antioxidants. Consequently, the mean of change from control in a population of subjects with varying cellular redox states likely would not be large, but the standard deviation would be large, leading researchers to conclude antioxidants were not effective in AD.

Redox proteomics studies in AD and its early stages

Redox proteomics, which was pioneered in our laboratory [6, 7, 50–56], leads to identification of excessively oxidized or HNE-bound proteins compared to those proteins in aged matched control brains. Redox proteomics methodology is based on protein separation, selection of oxidatively or nitrosatively modified proteins via sophisticated image analyses, trypsin digestion of these selected proteins, peptide clean-up, application of tandem mass spectrometry (MS/MS) methods to determine the peptide amino acid sequence, followed by interrogation of appropriate databases to identify the specific proteins since each protein has a unique amino acid sequence [6, 7, 50]. Application of redox proteomics to specimens from subjects with AD or MCI led to the identification of many brain proteins altered at different stages of AD progression [6, 7, 29, 32, 33, 51–70]. The reader is directed to the papers cited above and recent reviews [6, 7, 61] from our laboratory for experimental details and list of oxidized brain proteins identified. When classified into pathways, these oxidized proteins were in functional classes, who loss of functions were consistent with the clinical presentation, pathology, and biochemical alterations of AD, MCI, and preclinical AD (Table 1). For example, glucose utilization via glycolysis, the TCA cycle, and the mitochondrial electron transport chain was predicted to be compromised in AD based on redox proteomics-determined oxidative damage to key protein components of each major pathway involved in glucose utilization for ATP production. Our results and predictions are consistent with ^{18}F-glucose PET studies showing progressively decreased glucose utilization with increased stage of AD. In AD and MCI brain, diminution of ATP production following oxidative modification of these proteins is consistent with and likely contributes to: 1) loss of phospholipid asymmetry in cell membranes (that affects both membrane lipid integrity and function of transmembrane proteins and is a marker for apoptosis) [71]; 2) loss of synaptic remodeling associated with decreased neurotransmission and consequent decreased learning and memory [61, 72]; 3) decreased rate of neuronal mitochondrial anterograde and retrograde transport to and from energy-starved pre-synaptic terminals [73–77]; 4) decreased neuritic length (which would decrease efficiency of neuronal communication, clearly important in a disease associated with decreased cognition and memory [78]); and 5) elevated neuronal intracellular Ca^{2+} (that would both compromise glutamate neurotransmission processes and induce activity of several intracellular destructive enzymes, such as phospholipases, endonucleases, proteases, etc., thereby inducing both apoptotic and necrotic destruction of neurons) [79]; and many other aspects of AD associated with the clinical presentation, pathology, and biochemical alterations known in each stage of AD.

Table 1
Brain protein and/or pathway dysfunction as a consequence of oxidative damage in Alzheimer's disease or amnestic mild cognitive impairment revealed by redox proteomics*

Glucose metabolism, i.e., components of glycolytic, TCA, or ETC pathways
Anaplerotic "filling" reactions of the TCA cycle
Glutamate transport and removal, i.e., excitotoxicity
Synaptic function and neurotransmission
Proteasomal function
Membrane lipid abnormalities and cholinergic dysfunction
Shortening of neuritic length
Elevated Aβ production and tau hyperphosphorylation, and blocked exit from the cell cycle, the latter leading to apoptosis
Mitochondrial alterations
Cell signaling alterations
Apoptosis activation
Deficits in protein synthesis
Damaged antioxidant proteins

*See text for more details.

Some specific proteins are uniquely modified throughout all stages of AD, and I opine that this small subset of oxidized proteins may contribute to the progression of this disorder. Some of these specific proteins are involved in pathways for ATP production (enolase; ATP-synthase) or proteostasis (ubiquitin carboxyl-terminal hydrolyase L1 in the Ubuiquitin-Proteasome System; cathepsin D and V_0-ATPase for autophagolysosomal function in autophagy). Defects in glucose metabolism throughout the progression of AD were mentioned above. Autophagy is known to be decreased in AD [80, 81]. This loss of autophagic function throughout the progression of AD, which would lead to accumulation of cellular detritus and therefore cell death, also may be related to activation in the mammalian target of rapamycin (mTOR) pathway throughout the stages of AD [81], an activation that can be initiated by $A\beta_{42}$, among other means [82]. Our findings that oxidative dysfunction of such a small number of key proteins and pathways related to glucose utilization and removal of aggregated, damaged proteins occurs from the early stages of AD to late-stage AD are consistent with the notions that these proteins and pathways are critical

to AD progression and are potentially therapeutically targetable to slow this progression.

Recent studies from our laboratory reported results of investigations of brain from individuals with Down syndrome (DS) obtained as a function of age [81–92]. Though DS individuals exhibit intellectual disability from birth, often AD neuropathology and dementia occur in DS persons at approximately 40–50 years of age [93]. Oxidative stress and redox proteomics studies of brain from DS persons demonstrate changes similar to what is observed in AD brain [81–92]. In addition, activation of the mTOR pathway (with consequent dysfunction of autophagy and insulin signaling in brain), coupled with diminution of glucose metabolism and alterations in the proteostasis network of DS persons who have AD neuropathology and present with dementia, mirrors these characteristics of AD brain [81, 82, 88, 92]. The notion that insights into AD may be gained from study of DS persons is discussed in the section on Future Research Directions below.

Importance and implications of our research on AD

Throughout the above discussion, the importance and implications of our research on AD have been mentioned. Summarizing these discussion points regarding research from our laboratory:

- Oxidative stress is now considered by most AD researchers and clinicians to be a critical component of this dementing disorder and its earlier stages and a contributor to progression of AD.
- Neurotoxic oligomers of $A\beta_{42}$ were shown to be strongly associated with oxidative stress and correlated to protein oxidation and lipid peroxidation in AD and MCI brain and in *in vitro* and *in vivo* models thereof.
- Redox proteomics approaches led to the identification of oxidatively dysfunctional proteins and biochemical pathways, whose dysfunctions are consistent with the clinical presentation, pathology, and biochemical alterations of AD and MCI.
- A small subset of these redox proteomics-identified, oxidatively dysfunctional proteins and pathways are present from early stages to late-stage AD, suggesting their importance for the progression of this dementing disorder and potential therapeutic targets to slow or retard progression of AD.

PREDICTIONS FROM OUR LABORATORY OF FUTURE RESEARCH DIRECTIONS IN THE FIELD OF ALZHEIMER'S DISEASE

It is my opinion that future AD research will coalesce around several key processes/functions, the protection of which conceivably could slow, or ideally stop, progression of this devastating disorder. Below are some areas of future research in AD that I predict will be among these coalesced areas of research.

Plasma as a biomarker source

As noted above, neuropathology of AD is present approximately two decades prior to the onset of symptoms. Consequently, given the need to diagnose prodromal AD prior to the onset of symptoms, one area of predicted future research is the eventual identification of a reliable and unique set of biomarkers for the unequivocal diagnosis of AD from easily obtainable specimens. This notion is supported by our studies of beagles, in collaboration with the laboratories of Carl Cotman and Elizabeth Head [94]. Brain was isolated from 15-year-old beagle dogs (who have $A\beta_{42}$ deposition of the same amino acid sequence as humans), who for the preceding 3 years had been on a high antioxidant diet, exposed to a behaviorally enriched environment (to learn new tasks and thereby make new synapses), and given exercise. The oxidative stress levels in brain of such treated beagles were much lower and similar to those of much younger dogs and in marked contrast to unstimulated dogs fed dog chow. Moreover, the treated beagles had lower levels of $A\beta_{42}$ and performed in behavioral tests like younger dogs [94]. Assuming that these promising results are transferrable to humans, individuals with incipient AD identified by reliable and specific biomarkers could be placed on regimens of high antioxidant diets, exercise (as appropriate and able), and intellectual stimulation (i.e., crossword puzzles, learning a new language, taking up a new musical instrument, etc.). My opinion is that identification of biomarkers for prodromal AD well before onset of symptoms will be a critically important future research effort.

Along this line of thinking, our laboratory, working with that of Patrizia Mecocci, used mitochondria isolated from peripheral lymphocytes to demonstrate elevated oxidative stress and proteomics identification of key proteins of differential levels in both MCI and AD individuals [95, 96]. The elevation of oxidative stress in specific individuals was inversely

correlated with cognitive function assessed by the Mini-Mental State Examination and inversely correlated with levels of small soluble antioxidants [95, 96]. In addition, plasma, and to a lesser extent cerebrospinal fluid (CSF) (the hesitation due mostly due to the more invasive method of obtaining CSF), are fluids that may one day be a source of reliable and unique biomarkers. However, plasma has proteins at levels that span a range of 10^{14}, with a small number of proteins comprising 85–90% of total plasma proteins. Thus, in order to reliably measure proteins of much lower concentration, the major proteins (for example, albumin, IgGs, certain glycoproteins) need to be removed and separately analyzed. Using this approach in both fluids, in collaboration with Marzia Perluigi's laboratory, we reported significant changes in specific proteins using proteomics in AD and MCI [97–101]. At this stage of investigation, such studies are not sensitive enough nor specific enough for unequivocal biomarker-based diagnosis of MCI and AD. Continued improvement in separation technology will lead increased use of these methods for soluble protein-based biomarkers for AD and MCI in my opinion. The major plasma proteins, particularly albumin, also may serve as a biomarker, so, as noted above, the removed proteins also need to be investigated in my opinion.

Interestingly, plasma also has extracellular vesicles (EV) that emanate from neurons, and proteomics and other means of identifying proteins have shown early changes in neuronal protein composition in EVs from persons who would go on to develop AD [102, 103] or who have DS [104]. A good prediction of future research in the AD field is that EV-related research for biomarkers of neuronal origin will greatly expand.

Investigations of pathways identified as important in AD and MCI

Given that type 2 diabetes mellitus is a major risk factor for AD [105], studies of insulin signaling, which is inhibited following activation of the mTORC1 pathway [81], coupled with the role of mTORC1 activation in AD and MCI on inhibition of autophagy [80, 81], I predict that greater investigation of the mTOR pathway activation by $A\beta_{42}$ and other factors in AD and MCI brain and in patients will occur in the future. Currently, FDA-approved drugs, including rapamycin and metformin (among others), that inhibit mTOR are known. More studies to ensure no harm to patients arises, long-term use of these agents from likely will be investigated.

In the same vein, noting that oxidative stress and redox proteomics studies in AD and MCI brain and mitochondria from peripheral lymphocytes identified enolase, which is a pleitropic enzyme [106], and ATP synthase as oxidatively modified and dysfunctional proteins [96], I predict that research on finding ways to increase glucose metabolism in patients with early stages of AD will be pursued.

Oxidative stress

As discussed above, clinical trials in AD with small antioxidant compounds have been disappointing. However, as also discussed above, there may be key methodological and pedagogical reasons for these failures. Given that in aged beagle dogs use of high antioxidant diets coupled with intellectual stimulation and exercise led to improved cognition and dramatically reduced oxidative stress [94], I predict that research (and clinical practice) in AD in the future will emphasize this multi-pronged approach that was successful in reducing loss of cognition and significantly decreasing brain levels of $A\beta_{42}$. One particular aspect of this approach that likely will be increasingly emphasized in the future, I predict, will be research on the use of food rich in components that themselves induce a stress, to which cells respond to produce beneficial effects, so called cellular stress response [107] or hormesis [108]. One such cellular response is upregulation of Nrf-2-mediated phase 2 enzymes, such as heme oxygenase, gamma-glutaminylcysteine ligase, and Mn-superoxide dismutase (MnSOD) [107]. The notion of this prediction is that, in an analogous way that cancer therapy is evolving to stimulate the patient's own immune response to destroy cancer cells, hormetic approaches to cause the AD patient to upregulate her/his own antioxidant or other protective responses to the disease will be beneficial to the patient.

Studies of specific proteins in AD

Redox proteomics and western blot approaches from our laboratory have identified oxidative modification of many proteins, the functions of which are shown in Table 1 above. In the interests of space, I consider two proteins of particular relevance to future AD research (in addition to those mentioned elsewhere in this paper). One protein is peptidyl-prolyl cis-trans isomerase (Pin 1) [53, 109–111]. Pin 1 is a regulatory protein that controls the activity of target

proteins by binding to their phosphorylated Ser/Thr-Pro domains and converting the conformation of the proline residue from trans to cis and vice versa [109, 112]. This conformational change produces a very large structural change in the target protein, thereby regulating its activity [109]. Two such target proteins are AβPP, from which $A\beta_{42}$ is derived, and phosphorylated tau. In addition, protein phosphatase 2A, which removes phosphate moieties from hyperphosphorylated tau, also is a Pin 1-regulated protein. Consequently, oxidative dysfunction of Pin 1 would be related to two principal pathological hallmarks of AD: senile plaques and neurofibrillary tangles. Therefore, additional research on the role of Pin 1 in AD is predicted.

Biliverdin reductase-A (BVR-A), following action of heme oxygenase-1 (HO-1), converts bilverdin to bilirubin as part of the processes needed to rid the brain (and other organs) of toxic heme [113]. Moreover, at low levels, bilirubin is reportedly a powerful antioxidant and de-nitrifying enzyme [114]. However, BVR-A is a pleiotropic enzyme, having both reductive and kinase properties depending on which specific sites on BVR-A are phosphorylated by various kinases [113]. Among other enzymes, insulin receptor substrate-1, needed for transducing insulin signaling, is phosphorylated by BVR-A and may be critical to modulation of insulin signaling [115, 116], which is known to be defective in AD and MCI brain [81]. Our laboratory demonstrated that the HO-1/BVR-A system in brain is highly oxidatively modified and dysfunctional in AD and MCI [145, 146]. I predict that future research on AD will examine further the role of BVR-A in AD and MCI.

Statins

Inhibition of cholesterol synthesis by use of HMG-CoA reductase inhibitors (statins) has been suggested to reduce incidence of AD [119]. However, lowering of cholesterol levels in brain is not the reason for this proposed benefit, since many statins, including atorvastatin, do not cross the BBB [120, 121]. Future research into potential mechanisms by which statins may reduce incidence of AD might provide insights into underlying molecular processes in the disease itself. Consistent with this notion, our research using atorvastatin in the aged beagle dog model system for AD showed this statin greatly reduced oxidative stress and protected BVR-A in brain against oxidative and nitrosative modification, even though atorvastatin does not cross the BBB [120–125]. Atrovastatin also led to lower levels of Aβ and modulated various pathways to provide cognitive benefit [93]. I predict future research in AD will include studies on better understanding the molecular processes involved with non-BBB penetrable statins and potentially reduced incidence of AD.

Inflammation

Notwithstanding that there is ample evidence for inflammation in brain in AD [126], determining if this is causative or a result of AD remains the subject of investigation. As noted above, traditional small molecule anti-inflammatory compounds were not effective in clinical trials in AD. However, newer brain-accessible anti-inflammatory compounds likely will be the subject of future research in AD. Even if such compounds do not get at the primary cause of AD, decreasing neuroinflammation has its own benefits for AD patients and would justify this predicted future research.

Studies in Down syndrome

As discussed above, DS persons often develop AD-like neuropathology and dementia after the fourth decade of life [93]. Insights into the major treatable characteristics of trisomy of chromosome 21 are predicted to result from future research in DS, and, simultaneously, studies of the age-dependent changes in brain from DS individuals are predicted to give new insights into molecular processes and pathways of direct importance to AD, from which new therapies are predicted to result.

CONCLUSION

Given the rapidly aging Baby Boomer cohort in the United States numbering more than 70 million people, coupled with aging being the single most important risk factor for AD, a public health crisis is facing the US in terms of the enormous number of new AD patients predicted to arise over the next 20–30 years. The cost involved in caring for these people, including lost wages, is enormous, and might not be sustainable for the nearly two decade-long population bubble of Baby Boomers. Clearly, some intervention has to emerge to slow the onset of AD. Since the average lifespan from diagnosis to death is about eight years, delaying the onset of AD by five years immediately would cut by more than 50% the

number of persons with AD. Such an outcome might be a realistic first goal of future AD research. Consequently, a renewed dedication by government, the private sector, scientists, and physicians to achieving this goal is needed. For ordinary Americans, a greater commitment to lifestyle changes to minimize risk factors for developing AD will be required. All these efforts will necessitate more basic research into the causes and consequences of AD and development of disease-modifying agents and modalities.

This current paper gives the perspectives on the importance and implications of AD-related research done in the author's laboratory and his predictions of future directions of research into this ominous and challenging disorder. It has, and continues to be, our laboratory's great privilege to contribute to better understanding of some of the molecular processes involved in AD from which potential disease-modifying therapeutic strategies have emerged.

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Novel Key Players in the Development of Tau Neuropathology: Focus on the 5-Lipoxygenase

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Abstract. Tauopathies belong to a large group of neurodegenerative diseases characterized by progressive accumulation of hyperphosphorylated tau. Tau is a microtubule binding protein which is necessary for their assembly and stability. However, tau affinity for microtubules mainly depends on its phosphorylation status, which is the result of a delicate balance between kinases and phosphatases activity. Any significant changes in this equilibrium can promote tau fibrillation, aggregation, neuronal dysfunction, and ultimately neuronal loss. Despite intensive research, the molecular mechanism(s) leading to tau hyperphosphorylation are still unknown and there is no cure for these diseases. Development of an effective strategy that successfully prevents tau excessive phosphorylation and/or tau aggregation may offer a real therapeutic opportunity for these less investigated neurodegenerative conditions. Beside tau, chronic brain inflammation is a common feature of all tauopathies and 5-lipoxygenase, an inflammatory enzyme, is upregulated in brain regions affected by tau pathology. Recently, *in vitro* studies and preclinical investigations with animal models of tauopathy have implicated 5-lipoxygenase in the regulation of tau phosphorylation through activation of the cyclin-dependent kinase 5 pathway, supporting the novel hypothesis that this protein is a promising therapeutic target for the treatment of tauopathies. In this article, we will discuss the contribution of the 5-lipoxygenase signaling pathway in the development of tau neuropathology, and the promising potential that drugs targeting this enzyme activation hold as a novel disease-modifying therapeutic approach for tauopathies.

Keywords: 5-lipoxygenase, phosphorylation, tau protein, tauopathies

INTRODUCTION

Most of the very common neurodegenerative diseases are characterized by aberrant protein aggregation, subsequent intracellular precipitation and ultimately inclusion body formation. Hyperphosphorylation and aggregation of the microtubule-associated protein tau is the major pathological signature of a group of neurodegenerative disorders collectively referred to as tauopathies, which includes Alzheimer's disease (AD), Pick's disease, corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP) [1]. Clinically, tauopathies present a heterogeneous phenotype which may include both motor dysfunction and cognitive impairments. The tau protein is normally associated with microtubules and is necessary for their assembly and stabilization. However, when it becomes highly phosphorylated, its affinity for microtubule decreases and tau starts to polymerize into paired helical filaments (PHFs) which then accumulate and precipitate

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forming neurofibrillary tangles (NFTs) leading to microtubules disassembly, synaptic dysfunction and ultimately neuronal death [2]. Although several mutations of the gene coding for tau (MAPT gene) have been linked to rare familial forms of these disorders, tauopathies are mostly sporadic diseases and the etiological factors that trigger the pathological tau metabolism, initiate the abnormal conformation and intracellular accumulation in these cases are poorly understood [2].

In addition to tau pathology, neuroinflammation is another common feature of these disorders [3]. Previous research suggested that neuroinflammation is an early event and that activation of the inflammatory response exacerbates microtubule-associated protein tau (or tau) phosphorylation, tau pathology and cognitive deficits in several mouse models of tauopathy [3–5]. The 5-lipoxygenase (5LO) is a pro-inflammatory protein enzyme, widely expressed in the central nervous system (CNS) and is found to be upregulated in the brain of tauopathy patients [5]. Previous studies have provided evidence that this enzyme is significantly involved in age-associated neurodegenerative diseases. In fact, 5LO has shown to influence AD-like neuropathology, modulating both amyloid-β and tau metabolism in APP transgenic mice and in a mouse model with amyloid plaques and tau tangles [5–8]. Moreover, genetic or pharmacological modulation of 5LO activity influences memory impairments, synaptic dysfunction, and pathology and directly modifies tau phosphorylation in transgenic mouse models of tauopathies [5, 9]. Since altered tau metabolism and disrupted function have unequivocally been shown to be central to the neurodegenerative process in tauopathies, the prevention of tau phosphorylation and aggregation represents the main focus of the current drug development research approach in this specific area. However, the identification of a valid target able to efficiently affect these aspects of tau neurobiology and subsequent development of the associated neuropathology has proven to be rather challenging.

In this review, we will focus on new exciting findings which underscore the functional role that the 5LO signaling pathway plays, as a key regulator of tau phosphorylation and pathology, in the pathogenesis of these neurodegenerative diseases. Additionally, we will discuss the potential that 5LO has as a novel therapeutic target, and the promise that pharmacological inhibitors of this protein enzyme may have as a viable and disease-modifying treatment of human tauopathies.

TAU AND TAUOPATHIES

Tau protein

The microtubule associated protein tau, which in this article we will refer simply as tau protein, is encoded by the MAPT gene located on the human chromosome 17. In human, tau gene is composed of 16 exons and alternative splicing of exons 2, 3, and 10 that generate 6 isoforms that differ depending on the presence of the 3 or 4 conserved repeats (3R-tau, 4R-tau) through which tau binds to the microtubules [2]. In addition to alternative RNA splicing, tau can undergo extensive post-translational modifications including phosphorylation, glycosylation, glycation, ubiquitination, and cleavage or truncation. Due to its high content of serine and threonine residues, tau is a good substrate to a large number of protein kinases such as glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinase 5 (CDK5), mitogen activated protein kinases p38 MAPK, c-Jun amino-terminal kinase (JNK), ERK/MAPK, and different protein phosphatases such as PP1, PP2A, PP2B, and PP2C [10]. The phosphorylation status of tau is critical for the regulation of its function and subcellular localization and distribution. In fact, phosphorylation generally reduces or inhibits its microtubule binding property, while de-phosphorylation tends to restore its affinity for the microtubules (Fig. 1) [11]. In neurons, tau is found mainly in the axon associated to the microtubules where is required for microtubule assembly, axons growth and integrity and also for transport of molecular cargo to the synapses [2]. However, accumulating evidence suggest also a physiological role for tau at the synapse level and in the nucleus. Tau is present at both pre- and post-synaptic level and can modulate synaptic neurotransmitter receptor signaling and synaptic plasticity [2, 10]. Our understanding of tau function at the nuclear level is less clear but its interaction with several nuclear proteins seems to be important for nucleolar organization and genome stability [2, 10].

Tau in neurodegeneration

Previous studies on animal models of tauopathy have established that the development of tau excessive phosphorylation and tau pathology cause abnormal neuronal and synaptic function and cognitive deficits [12]. In fact, tau reduction has been shown to prevent neuronal loss, reverse pathological tau deposition, and to prolong survival in a transgenic

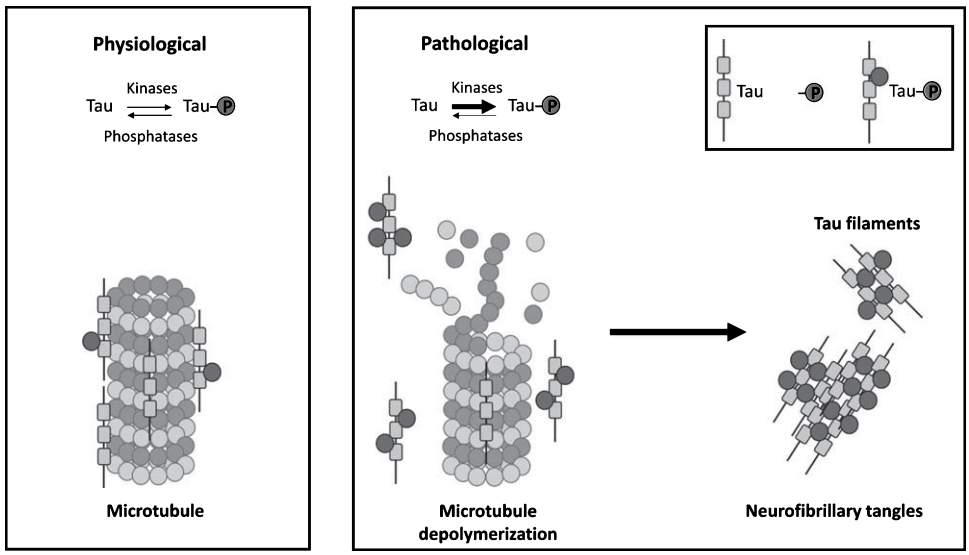

Fig. 1. The neurobiology of microtubule associated protein tau. In physiological conditions, tau is associated with microtubules to stabilize them and to keep healthy axonal transport and neurite outgrowth. However, in the brain of tauopathy patients, the disruption of normal phosphorylation events results in aberrant phosphorylation of tau which negatively affects its affinity for the microtubules and leads to microtubule destabilization. Once detached from the microtubules, hyper-phosphorylated tau tends to aggregate into paired helical tau filaments, which eventually become insoluble and precipitate inside the cells generating the neurofibrillary tau tangles.

mouse model of tauopathy expressing human tau mutant P301S, the P301S mice [13] revealing the crucial role of tau in mediating neurodegeneration. Notably, both brain imaging studies and CSF measures in AD patients have confirmed that the extent of tau neuropathology strongly correlates with the levels of dementia and memory loss [14]. More than 80 MAPT mutations have been linked to frontotemporal dementia with parkinsonism associated with chromosome 17 (FTD-17), CBD, and PSP but they only account for less than 5% of the total cases, thus what triggers tau abnormal phosphorylation and dysfunction in the sporadic forms of these disorders is still not clear [2]. The disruption of the equilibrium between tau kinases and tau phosphatases observed in tauopathies can significantly contribute to tau aggregation and toxicity. When highly phosphorylated, tau affinity for microtubule decreases and it starts to polymerize into PHFs which then accumulate forming NFTs leading to microtubules disassembly and tau mislocalization which impairs synaptic function (Fig. 1) [15]. Among all the kinases involved in tau phosphorylation, GSK-3β and CDK5 are probably the most investigated and today considered the most important ones, and their expression is in fact higher in the brain of tauopathies patients when compared with matched controls [16]. The role of these candidate kinases in the pathogenesis of tauopathy has been widely investigated in several relevant mouse models. For instance, when the P301L tau transgenic *JNPL3* mice are crossed with mice transgenic for the CDK5 activator p25, tau phosphorylation is increased at the putative CDK5 epitopes pThr181 (as recognized by the antibody AT270), pSer202, pThr231, and pSer396/pSer404 (AD2/PHF-1) and the number of NFT is five times higher when compared to the single transgenic mice confirming that this kinase plays an important role in this process [17]. In the normal human brain, tau has also been localized in both pre- and post-synaptic compartments where interact with the post-synaptic density protein 95/NMDA receptor complex [18, 19]. The potential mechanisms by which tau affects synaptic function is not clear; however, tau could play as a scaffold promoting interaction between the post-synaptic density protein 95/NMDA receptor complex and the tyrosine kinase *fyn*, thus regulating the NMDA-receptor signaling [2]. To this end, when tau is hyperphosphorylated, this interaction could be compromised leading to synaptic dysfunction. Beside phosphorylation, an imbalance in the 3R/4R ratio has been also observed in various tauopathies [2, 16]. Under normal physiologic condition, 3R-tau and 4R-tau are present in equal amount in the adult human brain. However, some recent studies have shown that 4R-tau isoforms, which generally have a greater microtubule-binding

affinity than the 3R-tau isoforms, are more efficient at promoting microtubule assembly. For this reason, today it is also believed that abnormal alternative splicing can also be involved in the promotion of tau dysregulated phosphorylation and ultimately pathological aggregation [16].

Tauopathies

Tauopathies are chronic neurodegenerative disorders clinically characterized by progressive loss of memory and learning ability (cognition), and impairments of motor functions. Although, several mutations of the gene coding for tau have been identified, the majority of tauopathies are mainly sporadic and thought to arise from the interaction of both environmental and genetic risk factors [11]. Currently, despite the major effort in the tauopathies research field, there are no effective therapies to cure or delay the progression of these disorders. From a pathological point of view, human tauopathies are quite heterogeneous syndromes [1]. In fact, although they all display hyperphosphorylation and accumulation of fibrillary tau in the CNS, tauopathies differ with respect to tau specific phosphorylation sites, cellular distribution, and isoforms found in the fibrillary lesions [1]. Furthermore, tauopathies can be categorized as primary or secondary depending on whether tau pathology is associated with other factors that may contribute to its development and progression of the disease or is the only pathological lesion found in the brains of these individuals [1, 2]. Primary tauopathies includes frontotemporal lobar degeneration, Pick's disease, PSP, and CBD. By contrast, AD represents the typical example of a secondary tauopathy. A further classification is based on the ratio of 3R/4R isoforms of tau which are essential for microtubules binding: PSP and CBD are 4R tauopathies; Pick's disease is 3R tauopathy; but AD typically displays an equal amount of the two isoforms (Table 1). Interestingly, no tau mutations have ever been identified in subjects with AD. Finally, different types of tau aggregates and cellular localization can be distinguished in these disorders. In AD, tau is mainly found in neurons as PHF, whereas in PSP and CBD, tau also accumulate in oligodendrocytes and astrocytes, in form of pre-neurofibrillary tangles in PSP but less filamentous in CBD [1, 2, 20].

Table 1
Most prevalent tauopathies and associated tau isoforms

Disease	Predominant isoform
Primary tauopathies	
PSP	4R
Argyrophilic grain disease	4R
Corticobasal degeneration	4R
Pick's disease	3R
FTDP-17	4R/3R
PEP	4R/3R
PDC Guam	4R/3R
Guadeloupean parkinsonism	4R
Secondary tauopathies	
Alzheimer's disease	4R/3R
Down's syndrome	4R/3R

Tau pathology and neuroinflammation

Increased neuroinflammation is strongly associated with NFT formation but whether it precedes or is driven by tau pathology *per se* is still not clear. Activated microglia and astrocytes co-localize with tau oligomers in the postmortem brain tissues of various human tauopathies including AD, FTD, PSP, and CBD [21, 22]. Moreover, the severity of brain inflammation correlates with disease progression, neuronal cell death, and cognitive impairments. What initiate tau phosphorylation and dysfunction is still not known but neuroinflammation seems to play an active role in this neurodegenerative process [3]. In fact, recent findings have demonstrated that microgliosis precede tangle formation in two mouse models of tauopathy: the hTau and P301S mice [5, 23]. Moreover, current research has shown that induction or inhibition of the inflammatory response can modulate tau pathology *in vivo*. The administration of lipopolysaccharide, the Toll-like receptor 4 ligand, can trigger tau hyperphosphorylation in the 3xTg AD mouse model [24], while administration of FK506, an immunosuppressant drug, decrease microgliosis in the P301S transgenic mouse model [23]. On the other hand, alterations in microglial phenotypes are also driven by tau dysfunction. Misfolded truncated tau is reported to activate the innate immune response via activation of the MAPK kinase pathway and to induce the release nitric oxide and other powerful pro-inflammatory cytokines (i.e., Interleukin-1β, Interleukin-6 and Tumor Necrosis Factor-α) [25, 26]. Finally, loss of tau in neurons and microglia provides protection against lipopolysaccharide-induced neurotoxicity, which under normal conditions triggers tau hyper-phosphorylation, tau pathology and ultimately cell loss and neurodegeneration [27].

The 5-lipoxygenase pathway

The 5LO protein is an enzyme that produces potent pro-inflammatory mediators such as

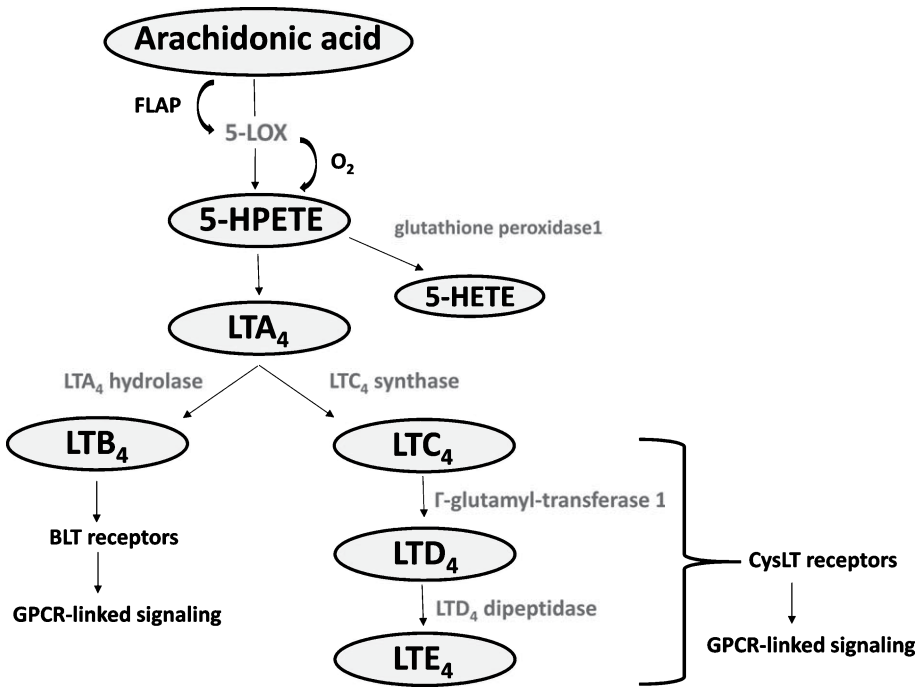

Fig. 2. The 5 lipoxygenase enzymatic pathway. Following cellular activation, 5-LO migrates from the cytosol to the nuclear membrane where it is able to interact with the 5LO activating protein (FLAP) and by oxidizing arachidonic acid on carbon 5 generates first 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which can then be converted to 5-hydroxyeicosatetraenoic acid (5-HETE), or leukotriene A4 (LTA4). LTA4 can be metabolized in leukotriene B4 (LTB4) by the action of a hydrolase, or into leukotriene C4 (LTC4) by the action of a synthase. LTC4 in turn can be transformed into leukotriene D4 (LTD4) by the γ-glutamyl transferase-1 and then into leukotriene E4 (LTE4) by the LTD4 dipeptidase enzyme. LTB4 action is mediated by bonding to the leukotriene B receptors (BLT1, and LTB2), whereas the LTC4, LTD4 and LTE4 action is mediated by their binding to the cysteinyl leukotriene receptors (CysLT). In both cases, the binding will elicit a GPCR-dependent intracellular signaling biological event resulting in immune activation and inflammatory responses.

leukotrienes by oxidation of the carbon in position 5 of free or esterified fatty acids, such as arachidonic acid [28]. Immediate product of 5LO enzymatic action is the 5-hydroxy-peroxi-eicosatetraenoic acid (5-HPETE), which is then metabolized into 5-hydroxy-eicosatetraenoic acid (5HETE) or leukotriene A4 (LTA4), depending on the cellular milieu. LTA4 is a substrate for the LTA4 hydrolase or LTC4 synthase generating LTB4 or LTC4, respectively. LTC4 can be then further metabolized by γ-glutamyl-transferase 1, and LTD4 dipeptidase to produce LTD4 and LTE4 (Fig. 2). Collectively LTC4, LTD4, and LTE4 are known as the cysteinyl-leukotrienes which signal through the activation of G-protein-coupled-receptors (GPCRs), cysteinyl leukotriene receptors (CysLT1, CysLT2) to modulate chemokine production, immune cell activation and inflammation [29]. The 5LO enzymatic activity is strictly dependent and regulated by the availability of another protein, 5LO-activating protein (FLAP), which is necessary for the delivery of arachidonic acid to 5LO at the nuclear membrane level and for 5LO full activation (Fig. 2) [29].

The 5LO is widely expressed in the cardiovascular system and CNS and its levels are upregulated with aging, a common risk factor for the development of both cardiovascular and neurodegenerative diseases. In fact, upregulation of 5LO is implicated in vascular inflammation, and myocardial infarction [4, 30]. Furthermore, this enzymatic pathway has been reported to increase after cerebral ischemia and variants of the ALOX5AP, the gene encoding the 5LO-activating protein, have been shown to be associated with a greater risk of stroke compared with matched controls [31, 32].

The role of 5LO in tauopathy

In the CNS, the 5LO is expressed by both neurons and glia cells [29, 34]. Interestingly, post-mortem studies have shown that 5LO levels are upregulated in AD and PSP patients, as well as in relevant mouse

models of AD and other tauopathies in areas of the brain more vulnerable to neurodegeneration, such as cortex and hippocampus [5, 33–35]. Following this discover, in recent years, our group has demonstrated that 5LO is a key player in the development of the full pathological phenotype of these neurodegenerative disorders [7, 8]. First, we showed that in a transgenic mouse model of AD with plaques and tangles, the 3xTg AD mice, 5LO pharmacological inhibition or genetic deletion reduces amyloidosis and tau pathology and restores memory loss and synaptic dysfunction. On the other hand, we saw that the same mice overexpressing 5LO display worsening of their memory performance, greater Aβ and tau phosphorylation accumulation, and increased neuroinflammation [8]. In particular, we demonstrated that the effect on tau phosphorylation was mediated by the activation of the CDK-5 kinase pathway [8]. In fact, 5LO inhibition or knockout specifically reduces not only expression levels of the two CDK5 coactivators, p35 and p25, but also CDK5 kinase activity *ex vivo*. By contrast, 5LO overexpression results in a significant increase in tau phosphorylation upon increased levels and activity of the CDK5 kinase pathway [5–8]. Additionally, inhibition of CDK5 activity prevents 5LO-induced phosphorylation of tau in an *in vitro* model of AD, thus confirming that 5LO acts through CDK5 to induce tau pathological changes.

However, since data in the literature have shown that Aβ itself can promote tau phosphorylation [36, 37], our observation did not address the important biological question of a direct or indirect (i.e., via Aβ) role that 5LO plays in tau phosphorylation. To this end, and to finally establish that 5LO effect on tau is independent form Aβ, the possible modulation of tau phosphorylation by the 5LO signaling pathway has recently been investigated in two different models of pure tauopathy: the hTau mice in which mouse tau is substituted by non-mutated human tau [11], and the P301S mice, carrying the MAPT P301S mutation which is associated with FTD [38]. In both models, 5LO is significantly upregulated in an age-dependent manner, and brain region-dependent fashion with hippocampus and cortex showing higher levels compared with controls, whereas no differences were detected when cerebellum of the two groups was compared [5]. The observation regarding the region-specific increase in 5LO levels confirm the findings we previously observed in AD brains. Interestingly, in P301S mice, levels of LTB4, an indirect measure of 5LO activity, are also significantly increased in both regions as early as 2 months of age, when tau pathology is not detectable yet. This finding suggests that the activation of this enzymatic pathway is an early event during the development of the phenotype in this mouse model of human tauopathy [5].

Further studies have demonstrated the beneficial effect of 5LO inhibition in hTau mice, using zileuton, a selective and specific 5LO inhibitor which is approved by the FDA for the treatment of asthma since it prevents leukotrienes formation. In this relevant tauopathy model, pharmacological targeting of 5LO enzymatic activity results in reduced levels of tau phosphorylation without affecting total tau expression levels [5]. In addition to these changes in phosphorylation, mice receiving zileuton display significant less insoluble tau and less immunoreactivity for MC1, an antibody which specifically recognizes pathological tau conformation [39], indicating that 5LO inhibition also prevents alteration of tau folding associated with PHF formation [5, 40]. Recently, to rule out the possibility of potential zileuton off-target effects, these data have been reproduced in the tau transgenic mice where 5LO was genetically deleted. In this study we showed that the absence of this enzyme is accountable for a significant reduction of tau phosphorylation at specific epitopes without influencing total tau expression [9]. As mentioned previously, this effect is mediated via inhibition of the CDK5 kinase pathway, as demonstrated by reduction of p35 and p25 expression, in tau mice lacking the 5LO gene. These results have been confirmed also using an *in vitro* approach, in primary neuronal cells stably expressing the whole human tau transgene (Fig. 3) [5, 9].

Pleiotropic effect of 5LO in tauopathy

Considering that tau neuropathology induces defects in synaptic plasticity, learning, and memory, it comes with no surprise that beyond tau phosphorylation, the 5LO enzymatic pathway is also implicated in modulating synaptic function and cognitive impairments. Behavioral and electrophysiological analyses of transgenic tau mice have shown that tau hyperphosphorylation and aggregation particularly affects synapses and causes significant reduction in the long-term potentiation responses [41]. However, pharmacological inhibition or genetic absence of 5LO enzyme rescues these brain functions. In fact, zileuton treatment as well as 5LO knockout in hTau and P301S mice results in better working and spatial

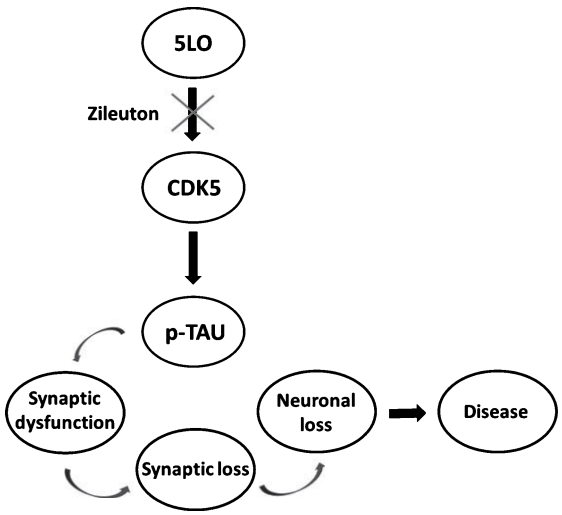

Fig. 3. Working model depicting the role that the 5-Lipoxygenase plays in the development of tau neuropathology. 5LO activation promotes tau phosphorylation via the CDK5 kinase and results in neuroinflammation, memory and synaptic dysfunction. Pharmacological inhibition of 5LO enzyme activity by zileuton by preventing activation of the CDK5 kinase reduces tau phosphorylation, synaptic dysfunction, neuroinflammation, neuronal loss and ultimately disease onset.

memory [5, 9] and protects from tau-induced synapsis dysfunction as measured by long term potentiation recording at both 10 and 120 minutes [5].

Together with these functional aspects of the synapse, manipulation of the 5LO pathway can also modulates the expression of several markers of synaptic integrity such as post-synaptic density protein 95, synaptophysin, and microtubule-associated protein 2 (MAP2) [42–45]. Thus, compared with controls transgenic tau mice chronically receiving the 5LO inhibitor, or born genetically deficient for the 5LO gene manifested significant improvements in these different synaptic proteins suggesting a modulatory ability of 5LO toward this important aspect of the tauopathy phenotype. Lastly, genetic deletion of the 5LO enzymatic pathway results in reduced activation of microglia and astrocytes, commonly found around NFTs-rich areas [46, 47] as demonstrated by a decrease in glial fibrillary acidic protein and cluster of differentiation 45 steady state levels and brain immunoreactivity to these proteins [5, 9]. Current animal models of neurodegenerative diseases have shown that the activation of the local inflammatory response is an early event and strongly influences the rate of disease progression suggesting that a viable therapeutic approach should potentially address both neuroinflammation and tau pathology. In this regard, 5LO represents an attractive target for the treatment of both aspects of the disease phenotype. Employing pharmacological inhibition, gene knockdown and overexpression of 5LO, these recent studies have validated the crucial role of this enzyme in the modulation of several aspects of tau pathology including tau phosphorylation, synaptic function and plasticity and memory in different mouse models of tauopathy [5, 9].

CONCLUSIONS

Tauopathies are a group of chronic neurodegenerative disorders characterized by progressive cognitive deficits and dementia. Aberrant phosphorylation of the microtubule associated protein tau has clearly been linked to the development of these neurodegenerative processes and several disease-causing mutations of the tau gene have been identified in some of these patients. However, tauopathies are mainly sporadic diseases, and the molecular and cellular mechanisms responsible for aberrant tau function in these cases are poorly understood. The prevention of increased tau phosphorylation and subsequent aggregation is currently the main focus of an intense drug development effort approach, but the research of a valid target able to directly modulate tau pathology and delay the progression of these diseases has failed so far. Beside tau, neuroinflammation is an active player in the neurobiology of these disorders. Growing evidence have shown that microgliosis and astrocytosis, two markers of cellular neuroinflammation, are strongly associated with NFTs deposition and neuronal toxicity, but whether activation of the inflammatory response is primary or secondary to the development of tau pathology is not clear.

Having established that 5LO, a pro-inflammatory enzyme, is upregulated in the brain of PSP patients and mouse models of tauopathy, and that knockout or pharmacological inhibition of 5LO activation is sufficient to reduce tau phosphorylation and to restore memory and synaptic function in several relevant models of the disease, this signaling pathway has recently emerged as a novel therapeutic target for the treatment of tauopathy. The successful completion of the initial step for the pre-clinical evaluation of a pharmacological inhibitor of this enzyme has now clearly paved the way for next step in this field: the investment in further research and development of this class of drugs (5LO inhibitors) as novel and potentially disease modifying agents with neuroprotective effects for human tauopathies.

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Findings from the Swedish Study on Familial Alzheimer's Disease Including the APP Swedish Double Mutation

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Abstract. This is a brief summary of the findings from the Swedish study on familial Alzheimer's disease (FAD). Similar to other FAD studies, it includes prospective assessments of cognitive function, tissue sampling, and technical analyses such as MRI and PET. This 24-year-old study involves 69 individuals with a 50% risk of inheriting a disease-causing mutation in presenilin 1 (*PSEN1* H163Y or I143T), or amyloid precursor protein (the Swedish *APP* or the arctic *APP* mutation) who have made a total of 169 visits. Our results show the extraordinary power in this study design to unravel the earliest changes in preclinical AD. The Swedish FAD study will continue and future research will focus on disentangling the order of pathological change using longitudinal data as well as modeling the changes in patient derived cell systems.

Keywords: Alzheimer's disease, biomarkers, cerebrospinal fluid, genetics, neuroimaging, neuropsychology

INTRODUCTION

Familial Alzheimer's disease (FAD) is a rare form of Alzheimer's disease (AD), caused by autosomal dominant mutations in one of three known genes, the amyloid precursor protein (*APP*) gene [1–3], the presenilin 1 (*PSEN1*) gene [4], and the presenilin 2 (*PSEN2*) gene [5]. FAD mutations are usually close to 100% penetrant, leading to AD with an early and predictable age at onset of first cognitive symptoms [6].

The Swedish FAD study was initiated at the Karolinska Institutet in 1993 and has now been ongoing for 24 years. The participants in the study belong to four Swedish families, each carrying a different mutation leading to FAD: the *PSEN1* H163Y mutation, the *PSEN1* I143T mutation, the Swedish *APP* mutation (*APPswe, KM670/671NL*), and the arctic *APP* mutation (*APParc, E693G*). A total of 69 individuals from these families have participated in the FAD study through the years, some repeatedly, amounting in 169 separate examination occasions. The clinical signs and symptoms in the participating families have been described in previous publications [7–9]. The age at onset of cognitive symptoms in these families is 54 ± 4 years for *APPswe* (based on 19 affected cases), 56 ± 4 years for *APParc* (based on 12 affected cases), 51 ± 7 years for *PSEN1 H163Y* (based on 11 affected cases), and 36 ± 2 years for *PSEN1* I143T (based on 5 affected cases).

The aim of the FAD study is to elucidate the pathological progress of AD through prospective collection of clinical and biomarker data from mutation carriers, with non-carriers from the same families serving as controls. The emphasis of the FAD study is on the

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preclinical stage of AD. Cognitively asymptomatic carriers of FAD mutations offer a unique opportunity to gather information on the preclinical stage of the disease, as they will develop the disease in the future with certainty and with a predictable age at symptom onset. Possible disease-modifying treatments for AD are now believed to be the most effective if initiated early in the course of the disease, preferably in the preclinical stage. Knowledge on the earliest detectable biomarker changes in this symptom free phase of AD is key when studying, and hopefully even applying, disease modification in the future.

All study procedures are in agreement with the Helsinki declaration and approved by the Regional Ethical Review Board in Stockholm, Sweden.

BIOMARKERS IN CEREBROSPINAL FLUID

The biomarkers amyloid-β $(Aβ)_{42}$, total tau-protein (t-tau), and phosphorylated tau-protein (p-tau) are routinely measured in the cerebrospinal fluid (CSF) of patients being evaluated for possible AD [10]. These markers offer support for diagnosing/excluding AD, with $Aβ_{42}$ typically decreasing and t-tau and p-tau increasing in AD. When measuring these three biomarkers in the CSF of 22 symptom-free participants from the FAD study (10 mutation carriers and 12 non-carriers), we observed a decrease in $Aβ_{42}$ 15–20 years before the expected onset of symptoms, while an increase in t-tau and p-tau was observed closer to the onset [11]. These findings are corroborated in other studies on the preclinical stage of FAD, that show a similar decrease in $Aβ_{42}$ in the CSF years before the onset of clinical symptoms [12–14]. CSF $Aβ_{42}$ is therefore a very early marker of AD pathology and useful both for early detection of the disease and potentially also for monitoring treatment response.

MAGNETIC RESONANCE IMAGING

Volumetric magnetic resonance imaging (MRI) is another well-established source of biomarkers in AD. The medial temporal atrophy score is widely used in the clinical setting to assess atrophy of the hippocampus [15–17]. A study by Bateman et al. detected a bilateral decrease in hippocampal volumes in carriers of FAD mutations 15 years before the expected onset of symptoms [12]. In another study on a different FAD cohort, Fox et al. reported similar results in 7 mutation carriers, albeit closer to the onset of cognitive symptoms [18]. When comparing 13 asymptomatic mutation carriers to 20 non-carriers from the Swedish FAD study, there was no significant difference in hippocampal volumes between the two groups. In this case, the mutation carriers had 9 years on average left to the onset of clinical symptoms. In the same study, however, there was a significant decrease in the volume of the left precuneus, left superior temporal gyrus, and left fusiform gyrus in the mutation carriers compared to the non-carriers [11].

Other modalities of MRI are also of interest in mapping the pathology of AD. By using diffusion tensor imaging, we observed white matter changes in the form of increased mean diffusivity in the left inferior longitudinal fasciculus, left cingulum and bilaterally in the superior longitudinal fasciculus in seven asymptomatic mutation carriers (compared to 20 non-carriers). When 3 symptomatic mutation carriers were included in the analysis, the affected areas became wider, suggesting early and progressive loss of myelination. In the same study, whole brain grey matter volume was analyzed and did not differ between the two groups [19].

Finally, 10 mutation carriers (3 of whom were symptomatic) and 13 non-carriers underwent resting-state functional MRI to assess functional connectivity in the default mode network (DMN). The DMN is a neuronal network that is active during rest and deactivates during active cognitive tasks. A decrease in functional connectivity has previously been observed in patients with mild cognitive impairment and dementia due to sporadic AD [20–22]. When all of the 10 mutation carriers were included in the analysis there was a decrease in functional connectivity in the right inferior parietal lobule, the right precuneus and the left posterior cingulate cortex. This decrease in functional connectivity did not reach significance when the symptomatic mutation carriers were excluded [23]. These findings suggest that amyloid and tau pathology interfere with neuronal and synaptic functions in the DMN, though this does not seem to be an early event in the disease cascade. However, lack of power to detect significant changes due to the small sample size may confound these results.

POSITRON EMISSION TOMOGRAPHY

In 2011, revised diagnostic criteria for AD were proposed by the National Institute on Aging – Alzheimer's Association workgroups [24]. This is

the first time that biomarkers are included in the diagnostic criteria for AD, but presently their use is generally only recommended for research purposes. The diagnostic criteria include biomarkers derived from the CSF as well as from imaging with positron emission tomography (PET) using ^{18}F-fluorodeoxyglucose (FDG) as well as PET ligands binding directly to amyloid [24].

In an FDG-PET study from 2009, 6 asymptomatic carriers of the *PSEN1* H163Y mutation were included, who were on average 20 years from expected symptom onset at baseline. The control group consisted of 23 non-carriers. Statistical parametric mapping revealed a trend of decreased thalamic glucose metabolism at baseline, which reached significance in the right thalamus at follow-up, 2 years later [25]. These findings suggest that metabolic changes are a very early event in AD, and this is in agreement with findings from other similar FDG-PET studies on carriers of FAD mutations [26, 27].

Inflammation has been implicated as a causative factor in AD and this has been supported by the discovery of reactive astrocytes surrounding amyloid plaques in brain tissue on autopsy [28–30]. ^{11}C-deuterium-L-deprenyl (DED) is a PET ligand that binds to monoamine oxidase B (MAO-B) on the outer mitochondrial membrane in astrocytes and its binding indicates reactive astrocytosis [31, 32]. When looking at DED binding in asymptomatic ($n = 6$) and symptomatic ($n = 3$) carriers of FAD mutations, as well as in patients with sporadic AD ($n = 7$) and mild cognitive impairment ($n = 11$), the highest level of DED binding was observed in the asymptomatic FAD mutation carriers. Conversely, DED binding was low in mutation carriers with cognitive symptoms. In the same study, an increase in the retention of ^{11}C-Pittsburg compound-B (PIB), an amyloid ligand, occurred early in the preclinical stage of FAD and predominantly in the anterior and posterior cinguli and the basal ganglia. These areas of increased PIB binding differed from the areas of increased DED binding [33]. This suggests that astrocytosis might be a response to non-fibrillar Aβ or even early plaque deposition and not to fibrillar Aβ as visualized by PIB binding. Furthermore, that astrocytosis occurs upstream of clinical symptoms and the formation of Aβ fibrils.

The findings from the multi tracer PET study described above were later replicated and further characterized with regards to temporality in a longitudinal study using the same tracers [34]. By using linear mixed-effects models, fibrillary Aβ plaque deposition was first observed in the striatum of asymptomatic FAD mutation carriers 17 years before the expected symptom onset. At about the same time, astrocytosis was significantly increased and then steadily declined. Diverging from the astrocytosis pattern, Aβ plaque deposition increased with disease progression. Glucose metabolism steadily declined from 10 years after initial Aβ plaque deposition. The prominent initially high and then declining astrocytosis in FAD mutation carriers, contrasting with the increasing Aβ plaque load during disease progression, suggests that astrocyte activation is most prominent in the early stages of AD pathology.

NEUROPSYCHOLOGY

Signs of cognitive decline through repeated neuropsychological tests are yet another biomarker of interest for early detection of AD. In 2017, Almkvist et al. published the results of neuropsychological assessments of the participants in the Swedish FAD study [35]. The participating mutation carriers were in different stages of FAD, from 28 years before the expected onset of symptoms until 12 years past the expected onset, spanning four decades of the disease. The age at symptom onset is a recurring concept in studies on FAD and is derived from the average age at onset of the first subjective cognitive symptoms in affected individuals in each FAD family. This family specific age at onset is currently widely used in research to estimate the expected onset age of asymptomatic mutation carriers [36].

The study by Almkvist et al. included 35 mutation carriers and 44 non-carriers who underwent a comprehensive neuropsychological assessment. A decline in performance on the Rey Auditory Verbal Learning test, an episodic memory test, was observed in the mutation carrier group 10 years prior to the expected symptom onset. This change was closely followed by a decline in performance in tests assessing executive function (Digit Symbol) and visuospatial ability (Block Design). These results are of particular interest as they imply that an objective decline on neuropsychological tests, covering several areas of cognition, precedes the subjective symptoms experienced by the patient.

CONCLUDING REMARKS

A hypothetical model of biomarker changes in the preclinical stage of AD was published by Jack et al.

in 2010 [37]. According to this model, the earliest changes are observed in biomarkers reflecting the accumulation of Aβ, both in the CSF and on amyloid PET. These changes are followed by changes in biomarkers reflecting tau pathology (in the CSF and on FDG-PET). At the end of the preclinical stage, structural changes on MRI can be observed and shortly thereafter a decline in memory, heralding the onset of mild cognitive impairment.

The earliest biomarker changes observed in the asymptomatic mutation carriers from the Swedish FAD study were a decrease in CSF Aβ as well as increased binding of PIB on PET, corresponding nicely to the model proposed above. Interestingly however, an increase in DED binding on PET and a decrease in thalamic glucose metabolism on FDG-PET, reflecting inflammation and neuronal death, were early events as well. These changes on PET were further characterized in a longitudinal study showing that DED and PIB binding diverged as the preclinical stage progressed, with a decrease in DED binding and an increase in the uptake of PIB as the age at symptom onset approached. Later in the preclinical stage, around 10 years from symptom onset, there was a decline in episodic memory and atrophy was detected in several areas in the left cerebral hemisphere on volumetric MRI. White matter changes on MRI were also observed in the preclinical stage. Finally, an increase in CSF tau and p-tau was observed close to the onset of symptoms. This places the CSF markers of tau pathology downstream of both volumetric MRI and cognitive decline assessed by neuropsychological tests.

The sample size in the FAD study is small, due to the rarity of this disease, which detracts somewhat from the robustness of the acquired data. Also, the data presented here is mostly cross-sectional which reduces the certainty of our conclusions on the temporality of events in the preclinical stage of AD. However, the results from this valuable group of patients add to the current base of knowledge on biomarker changes in this stage of the disease and warrant further investigation in larger cohorts of FAD mutation carriers as well as in sporadic AD. Our future goals are to use the longitudinal collected data and make comparisons between the biomarkers and thereby provide further insights into the chain of pathological changes in preclinical and clinical stages of AD. Furthermore, developing biomarkers in serum and plasma will be an important goal to replace or complement the more invasive and technically demanding CSF and PET based assessments.

Finally, as part of the Swedish FAD study, all subjects provide fibroblasts and current studies have shown promising results regarding the potential use of patient derived cells for both basic scientific studies of cellular mechanisms of neurodegeneration as well as a possible tool for treatment [38]. We have thus also initiated a cell modeling program where we hope to elucidate some of the possible mechanisms of reduced penetrance in PSEN1 H163Y mutation carriers as well as study the effects of autophagy dysfunction in neurodegeneration as observed on autopsy of AD and other tauopathies

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Untold New Beginnings: Adult Hippocampal Neurogenesis and Alzheimer's Disease

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Abstract. Neurogenesis occurs in a limited number of brain regions during adulthood. Of these, the hippocampus has attracted great interest due to its involvement in memory processing. Moreover, both the hippocampus and the main area that innervates this structure, namely the entorhinal cortex, show remarkable atrophy in patients with Alzheimer's disease (AD). Adult hippocampal neurogenesis is a process that continuously gives rise to newborn granule neurons in the dentate gyrus. These cells coexist with developmentally generated granule neurons in this structure, and both cooperative and competition phenomena regulate the communication between these two types of cells. Importantly, it has been revealed that GSK-3β and tau proteins, which are two of the main players driving AD pathology, are cornerstones of adult hippocampal neurogenesis regulation. We have shown that alterations either promoting or impeding the actions of these two proteins have detrimental effects on the structural plasticity of granule neurons. Of note, these impairments occur both under basal conditions and in response to detrimental and neuroprotective stimuli. Thus, in order to achieve the full effectiveness of future therapies for AD, we propose that attention be turned toward identifying the pathological and physiological actions of the proteins involved in the pathogenesis of this condition.

Keywords: Adult hippocampal neurogenesis, Alzheimer's disease, granule neuron, GSK-3β, morphology, neuroprotection, tau

ALZHEIMER'S DISEASE: A FEW WORDS ABOUT TWO OF THE MAIN PLAYERS, GSK-3β AND TAU

Alzheimer's disease (AD) is the most common type of dementia in industrialized countries. Although the etiology of the disease remains to be fully elucidated, age, genetic, lifestyle, and environmental factors appear to confer higher susceptibility [1]. AD is characterized by progressive neuronal cell death and atrophy of specific brain areas, including the entorhinal cortex (EC) and the hippocampus, and by a marked impairment of episodic memory [2, 3]. The most relevant histopathological hallmarks of the disease are extracellular senile plaques made of amyloid-β (Aβ) protein, and neurofibrillary tangles, which are formed mainly by hyperphosphorylated tau

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protein. Aβ and tau have been largely considered to be the cornerstones of AD pathogenesis [4]. Moreover, *in vitro* [5] and *in vivo* [6] studies showed that Aβ exerts its detrimental actions by activating a key kinase, namely glycogen synthase kinase 3β (GSK-3β) [5, 7], thus revealing this kinase as an important player in the amyloid cascade. GSK-3β is the main kinase that phosphorylates tau [7, 8]. Moreover, an increase in GSK-3β activity has been observed in the brains of AD patients [9]. These data have confirmed GSK-3β as a cornerstone of AD pathogenesis and support the notion that this kinase is a crucial molecular link between Aβ and tau [7, 8, 10–13]. Indeed, a transgenic animal model that overexpresses GSK-3β in the hippocampus (namely GSK-3β-oe mouse), generated in our laboratory, has been used to model the cellular and behavioral alterations that occur in AD. In these mice, conditional overexpression (OE) of GSK-3β results in impaired spatial memory and increased tau phosphorylation in the hippocampus [14–16].

The present work is focused on the hippocampal region, given the relevance of this brain region in AD pathogenesis. It revises the available literature on the effects of GSK-3β and tau dysregulation on a specific neuronal population of this region, namely granule neurons. Special emphasis is placed on the therapeutic potential of diverse interventions aimed to increase hippocampal plasticity in AD.

A FEW WORDS ABOUT ADULT HIPPOCAMPAL NEUROGENESIS (AHN)

Adult neurogenesis is an infrequent phenomenon in the mammalian brain. Under physiological conditions, only a limited number of human brain regions, including the hippocampus [17], the sub-ventricular zone [18], and the striatum [19], experience this process throughout lifetime. AHN has attracted considerable interest mainly because of the involvement of the hippocampal region in learning and memory and the marked atrophy of this structure in patients with AD [20]. In the hippocampus, adult neurogenesis continuously gives rise to newborn granule neurons (NGNs) throughout life [17, 21–23]. These newly generated cells derive from a special population of radial glial-like precursor cells [24], which undergo several rounds of asymmetric division and generate transiently amplifying progenitors [25]. These cells actively divide in specialized neurogenic niches and go through several development stages before reaching full maturity [26, 27]. During maturation, NGNs extend their dendritic trees through the granule and the molecular layers (GL and ML) of the dentate gyrus (DG), where they receive afferent innervation from the EC [28] and inhibitory Parvalbumin$^+$ interneurons [29]. Moreover, they extend their axonal projections toward the CA3 [30] and the CA2 [31] hippocampal subfields. Importantly, numerous aspects of newborn neuron generation in the hippocampus, including proliferation, maturation, and survival rates, are altered in animal models of AD and in patients with this disease [32, 33].

ORCHESTRATION OF AHN BY GSK-3β AND TAU

Regulation of the rate of AHN

In the DG, GSK-3β increases the proliferation of neuron precursors and prevents them from acquiring a neuronal fate [34]. Moreover, GSK-3β overactivation increases the apoptosis of mature granule neurons and blocks the differentiation of neuroblasts [35]. Interestingly, an increased number of Doublecortin (DCX)$^+$ neuroblasts is observed in GSK-3β-oe mice [35]. In this regard, the successive stages of AHN are featured by the expression of specific markers, and a stereotyped pattern of expression is thought to drive the maturation of these cells [25, 26, 36]. For instance, DCX expression is switched off at 3-4 weeks of cell age under physiological conditions. However, we found that its expression was aberrantly prolonged until the sixth week of cell age in GSK-3β-oe mice [35], thus rendering an increased number of neuroblasts whose maturational progression was blocked. As will be further discussed, this blockade has important consequences for NGN functionality in GSK-3β-oe mice.

As previously mentioned, tau is one of the main downstream targets of GSK-3β and is considered a capital regulator of AHN [37, 38]. In this regard, both the expression and the post-transcriptional modifications of tau are tightly regulated during the maturation of NGNs [37–39]. For instance, the tau isoform featured by the presence of three-repeat microtubule-binding domains (namely, 3R-tau) is transiently expressed during the NGN neuroblast stage [38]. In rodents, individual NGNs show the highest expression of 3R-tau at 2 weeks of cell age [39], and the expression of this molecule is maintained until 4 weeks, a time point at which 3R-tau

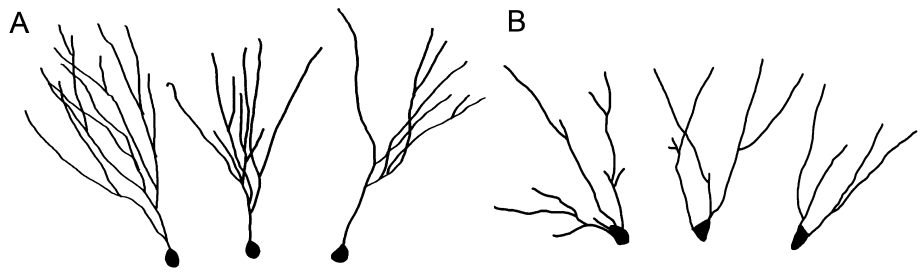

Fig. 1. Tracings of Golgi-stained granule neurons of control subjects (A) and patients with Alzheimer's disease (AD) (B). It should be noted how granule neurons of control subjects display a single primary apical dendrite emerging from the soma, thus resembling a "Y-shape", whereas granule neurons of AD patients show several primary apical dendrites that are poorly branched in the distal domains, thus acquiring a "V-shape".

is replaced by the four-repeat microtubule-binding domain form of the protein (4R-tau) [38]. By using an antigen retrieval protocol, we showed that 3R-tau is a transient marker of NGN axons [39]. Noteworthy, this isoform of tau confers the cytoskeleton with greater plasticity than the 4R-tau isoform and the expression of 3R-tau is coincident with the period of time in which NGNs exhibit highest plasticity [40]. Although the 3R-tau/4R-tau ratio in humans and rodents appears to differ [41], the absence of reliable human 3R-tau markers for immunohistochemistry determinations has hindered the detailed study of these regulatory mechanisms in the context of human AHN to date. However, it has been proposed that an imbalance in the aforementioned ratio underlies certain neurodegenerative diseases [42]. This notion deserves further exploration in the context of AD.

Despite the predominant role that tau is assumed to play in AHN regulation, tau knock-down does not cause alterations in the proliferation, differentiation, or survival rates of neural precursors in the DG *in vivo* [43]. In this regard, compensatory mechanisms exerted by other microtubule-associated proteins (MAPs), such as MAP-1A and MAP-1B [44, 45], occur in the absence of tau. In contrast to the apparent absence of alterations showed by tau knock-out (tau-/-) mice regarding the basal rate of AHN, we and others have demonstrated that various animal models of tauopathies in which pathogenic forms of tau are overexpressed exhibit marked alterations in AHN [37, 46]. In this regard, the overexpression of a pathogenic form of the protein is not only characterized by its lack of physiological functions but also by the gain of toxic functions. A recent study by our group also revealed that stereotaxic injection of a soluble form of tau has devastating effects on the structural plasticity of granule neurons and impairs pattern separation ability [47]. These data might be relevant for the field of neurodegenerative disorders, since they contribute to shedding light on novel pathological roles played by distinct tau species *in vivo*.

The "V-shape" phenotype of newborn granule neurons in AD

As previously mentioned, NGNs go through a multi-stage development process before they reach maturity [25]. Although fully mature newly and developmentally generated granule neurons were initially described to be undistinguishable [48], growing evidence indicates that they show both functional and anatomical differences [49]. Regarding the latter, these two types of granule neurons differ in their positioning with respect to the GL [49, 50]. In this respect, NGNs are located in the inner third of the GL, whereas developmentally generated granule neurons are found in the two outer thirds of this layer [50]. Moreover, both types of cell show morphological particularities worthy of further discussion, the most remarkable difference being the number of primary apical dendrites. Under physiological conditions, most newly generated granule neurons show a single primary apical dendrite, which is extensively branched in the ML, thus resembling a "Y- shape" [49]. In contrast, developmentally generated granule neurons show several primary apical dendrites emerging from the soma [51]. In contrast to the scenario observed under physiological conditions, we showed that GSK-3β OE causes a dramatic change in the morphology of NGNs by triggering the presence of several primary apical dendrites, thereby conferring them a "V-shape" [16]. The relevance of this observation lies in the fact that the same morphology is observed in the granule neurons of AD patients [16]. Representative neuronal tracings corresponding to Golgi-stained granule neurons belonging to

control subjects and AD patients are shown in Fig. 1. To the best of our knowledge, our work was the first to describe the occurrence of this phenomenon in AD patients; however, subsequent studies by other authors have revealed a similar "V-shape" phenotype, including the appearance of several primary apical dendrites in various pathological conditions [52–55]. Further efforts should address the molecular mechanisms involved in the appearance of this particular phenotype. In this regard, several molecules associated with the cytoskeleton may drive the development of dendritic branches and should be further explored in the context of neurodegenerative diseases [56].

Despite the observed marked effects of GSK-3β OE on the morphology of mouse NGNs, a critical question for the AD research field is whether these effects are a *cell-autonomous* consequence of GSK-3β OE or whether they represent a *non-cell-autonomous* indirect effect derived from massive cell death or neuroinflammation. This question is particularly relevant in the context of AHN, given that the promoter used to drive GSK-3β OE, namely *CamKII*, is active only in mature cells [16]. In order to address this point, we developed an innovative system to selectively drive GSK-3β OE in our target cells, namely NGNs [57, 58]. This system takes advantage of the capacity of retroviruses to exclusively transduce proliferating cells and of the fact that proliferation is restricted mostly to NGNs in the DG [30]. This novel system is based on the stereotaxic injection of a retrovirus encoding the reverse Tetracycline activator (rtTA) element into the hippocampus of tetR-GSK-3β mice. These animals carry a bi-directional Tetracycline repressor (TetR) promoter followed by a GSK-3β cDNA in one direction and a cDNA encoding β-Galactosidase (β-Gal) fused to a nuclear localization signal in the other. By using this methodology, GSK-3β OE occurs only in those NGNs infected by the retrovirus and after the administration of Doxycycline. Thus, our novel methodology has three major advantages over traditional transgenic mouse systems. The first is the use of an rtTA element instead of the classical tTA element. This approach renders a tet-ON system in which GSK-3β OE is selectively triggered by the administration of Doxycycline—a feature that allows precise temporal control of GSK-3β OE. The second advantage is that the system allows tight regulation of the age and type of cell populations that overexpress this protein by means of Doxycycline administration during the desired periods. Finally, the system allows the double monitoring of GSK-3β OE by means of two reporter proteins, namely EGFP (encoded by the retroviral genome) and β-Gal (encoded by the murine genome). As previously mentioned, this methodology allowed us to achieve rapid and selective GSK-3β OE in the NGNs infected by the retrovirus [57]. Using this strategy, we demonstrated the *cell-autonomous* character of the alterations triggered by GSK-3β OE in NGNs, which showed a "V-shape" phenotype only under tet-ON conditions.

This system shows great versatility and potential utility in the field of neurodegenerative diseases, since it has proved highly suitable for the study of the *cell-autonomous* effects of other pathogenic proteins involved in these conditions [58].

Synaptic integration of newborn granule neurons

Although the functional consequences of the morphological alterations previously described remain to be fully elucidated, we have proposed that a reduced dendritic mass in the ML decreases the afferent connectivity of NGNs by reducing the likelihood of distal dendrites receiving afferent contacts from the EC. This hypothesis is strongly supported by the clear detrimental effect of GSK-3β at the synapse [59]. In this regard, long-term depression downregulates long-term potentiation through GSK-3β activation [60, 61], a phenomenon which is followed by synapse elimination.

By using a PSD95:GFP-expressing retrovirus [62], we demonstrated that the NGNs of GSK-3β-oe mice exhibit a marked reduction in the number and size of postsynaptic densities (PSDs), thus revealing impaired afferent connectivity [16, 63]. Special mention should be given to the important consequences of selective impairment of the synaptic integration of NGNs on the whole trisynaptic circuit. In this regard, during the period in which NGNs are *young and excitable* [40], they are thought to play a key role in information processing along the hippocampal circuit [64, 65]. In fact, it has been proposed that newly and developmentally generated granule neurons cooperate to create an accurate and complex representation of new memories. Developmentally generated granule neurons are believed to be involved mostly in pattern completion (an ability based on generalization) [66], whereas the lower activation threshold and higher excitability of NGNs favor their involvement in hippocampal pattern separation (a phenomenon consisting of the production of two differentiated outputs in response to very similar inputs) [67, 68]. The continuous re-definition of

this elegant hypothesis proposed by Sahay [68] and McHugh [67] is continuously revealing the existence of additional complex orchestration mechanisms. For instance, competition phenomena between newborn and developmentally generated neurons to establish synaptic contacts with afferent fibers from the EC may also drive the intricate communication between this structure and the DG [69, 70]. This notion has been further supported by an elegant study by Sahay et al., in which the authors observed that reducing the connectivity of mature granule neurons dramatically increases the survival, maturation, and number of NGNs and maturity of synaptic connections of these cells [71]. These observations may have important consequences in the context of pathological conditions involving an imbalance between the synaptic integration of newly and developmentally generated granule neurons. In this regard, we found a similar imbalance in a murine model of AD overexpressing GSK-3β (GSK-3β-oe mice) in the hippocampus [51]. Granule neurons of these animals show a generalized reduction in the number of dendritic spines and synaptic contacts [51]. However, detailed morphometric analysis of these structures revealed that NGNs show a reduction in PSD volume (which in turn revealed a decrease in the synaptic strength of their afferent connections), whereas the dendritic spines of developmentally generated neurons show a marked enlargement (thereby suggesting a strengthening of the afferent connections of these cells). According to the competition hypothesis, these findings suggest the selectively impaired synaptic integration of NGNs in this animal model of AD. On the basis of the higher plasticity conferred by these cells to the hippocampal circuit, we have proposed that their reduced synaptic integration favors greater stability of old synaptic connections and a generalized lack of plasticity at the hippocampal level. Noteworthy, GSK-3β-oe mice exhibit alterations in hippocampal-dependent behaviors requiring high behavioral flexibility, such as in pattern separation [55].

There is intense debate in the field as to whether competition phenomena established between newly and developmentally generated NGNs are a physiological mechanism aimed to compensate the reduced capacity of the oldest neurons to cope with insults, or whether the natural *purpose* of NGNs is to gradually replace the oldest cells in order to favor their withdrawal from the hippocampal circuit [72, 73]. Nevertheless, the observation of these competition phenomena acquire particular relevance in the context of neurodegenerative diseases, given that impairments in pattern separation and in AHN have been found in AD patients and in animal models of this disease [32]. These observations and the extraordinary regenerative potential of AHN have placed this process in the spotlight of therapeutic strategies aimed to tackle neurodegenerative diseases.

By using the same retroviral approach previously mentioned, we demonstrated that GSK-3β OE increases tau phosphorylation in single NGNs in a *cell-autonomous* manner, thus revealing tau as a key mediator of the alterations in NGN functionality caused by GSK-3β OE. In this regard, despite the classical association of tau with the axonal compartment, growing evidence strongly supports the notion that this protein is present and involved in various functions of the somatodendritic compartment. In 2011, Ittner et al. [74] elegantly demonstrated the participation of tau in cortical synapses. In that study, the authors showed that tau depletion is neuroprotective against synapse destabilization caused by the presence of Aβ. More recently, these authors have demonstrated that tau phosphorylation finely tunes the synaptic roles played by tau [75]. It has been proposed that tau modulates the synaptic localization of *Fyn* kinase, which favors synapse stabilization [74]. On the other hand, tau phosphorylation by GSK-3β increases tau aggregation and impedes several of its actions at the synapse. A detailed schematic diagram showing these regulatory mechanisms is shown in Fig. 2. In agreement with previous observations in other neuronal populations [74], our data reveal that the absence of tau impairs the synaptic integration of NGNs [43]. These alterations are particularly apparent in the most distal part of the dendritic tree, where the NGNs of tau-/- mice show the most drastic reduction in the number of PSDs, accompanied by a marked decrease in the volume of these structures [43]. Importantly, these data indicate that the synaptic integration of NGNs in tau-/- mice is impaired, as a result of a reduction in the connectivity of the outer parts of their dendritic trees with afferent fibers from the EC.

Nevertheless, the general consensus in the field is that the absence of tau confers a certain degree of neuroprotection in response to exposure to various toxic agents. In this regard, we demonstrated, for the first time, that the absence of tau conferred NGNs with extraordinary resistance to stress [43]. However, our results also showed that the absence of tau completely blocked the stimulatory actions exerted by environmental enrichment (EE) on NGNs. Of note, EE is one of the most potent positive regulators of AHN

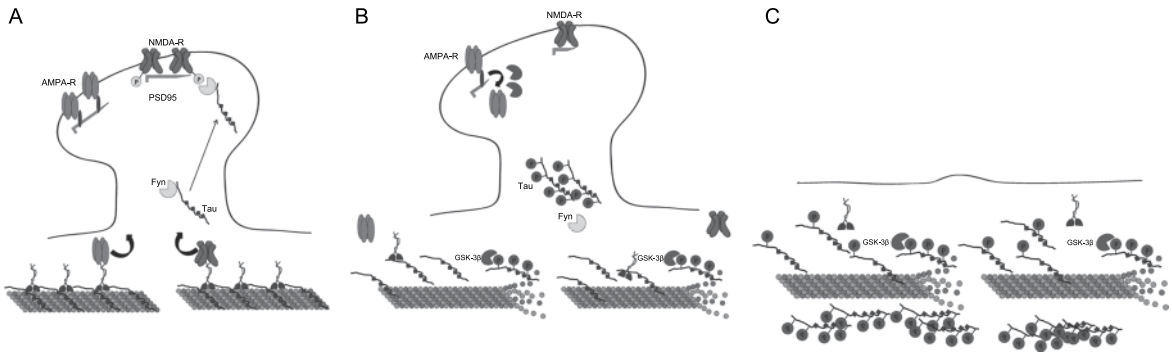

Fig. 2. Schematic model proposed to explain the mechanisms through which GSK-3β and tau regulate the synaptic integration of granule neurons. A) Under basal conditions, tau plays a central role at the synapses through its interaction with Fyn kinase. On the other hand, tau stabilizes microtubules, thus allowing the dendritic transport of synaptic receptors to the synapses, where they are stabilized through their interactions with the scaffold protein PSD95. GSK-3β exerts tau-dependent and -independent regulatory actions at the synapse. B) Under pathological conditions, GSK-3β overexpression increases tau phosphorylation. This phenomenon decreases tau affinity to bind microtubules, which are destabilized. On the one hand, this compromises dendritic transport. On the other hand, tau phosphorylation triggers its aggregation, which further impairs cell function. In addition, synapses are destabilized by tau-dependent and tau-independent mechanisms. C) The chronic maintenance of these pathological conditions leads to the disappearance of the synapse.

[76]. It increases the survival, maturation, and plasticity of NGNs in WT mice [77]. However, we did not observe these stimulatory effects in tau-/- NGNs [43]. Our results therefore indicate that tau depletion has a negative impact on neurodegenerative diseases by further decreasing the plasticity of especially sensitive neuronal populations. These data should be taken into account during the development of strategies aimed to ameliorate neurodegeneration caused by aberrant forms of tau. Special emphasis should be placed on identifying the physiological roles played by the various species of tau in specific neuronal populations, given that not all of these functions can be compensated by other MAPs.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Our current perception of AHN is continuously being modified by the addition of new brush-strokes that illustrate particular aspects of the orchestration mechanisms underlying AHN. Given the enormous complexity of the regulatory mechanisms driving the synaptic integration of NGNs into the pre-existing trisynaptic circuits, future efforts should be focused on unraveling the fine boundary between the neuroprotective and detrimental actions exerted by GSK-3β and tau. In this regard, we have observed that the absence of tau blocks the stimulatory actions of EE on NGNs. On the other hand, we have found that GSK-3β OE prevents some of the stimulatory actions of EE and physical exercise on NGN connectivity. Thus, both an increase in and blockade of the activity of certain proteins, such as GSK-3β or tau, may have equivalent detrimental effects on the structural plasticity of NGNs, a crucial cell population that shows marked alterations in animal models of AD models and patients with this condition. Hence, to achieve effectiveness, therapeutic strategies aimed to ameliorate the symptoms or to prevent the progression of AD should be particularly careful in the choice of pathological target and should ensure the maintenance of the essential physiological functions of these proteins.

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Our Tau Tales from Normal to Pathological Behavior

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Abstract. The microtubule associated protein tau in a hyperphosphorylated form was identified as the building block of the filamentous aggregates found in the neurons of Alzheimer's disease (AD) patients. In the abnormal state, hyperphosphorylated tau from AD brains (AD P-tau) was unable to promote microtubule assembly and more importantly, it could inhibit the normal activity of tau and other MAPs. AD P-tau was able to disrupt preformed microtubules and, by binding to normal tau, turn the latter into an AD P-tau like molecule. AD P-tau toxic behavior was prevalent in the soluble form and it was lost upon dephosphorylation. Mutations on tau associated with disease, e.g., R406W in frontotemporal dementia with Parkinsonism linked to chromosome 17, altered its conformation to make it a better substrate for kinases. Using phospho-mimetics, it was found that the minimum phospho-sites necessary to acquire such a toxic behavior of tau were at 199, 212, 231 and 262, and tau pseudophosphorylated at those sites in combination with R406W was named Pathological Human Tau (PH-Tau). PH-Tau expressed in cells had similar behavior to AD P-tau: disruption of the microtubule system, change in the normal subcellular localization, and gain of toxic function for cells. In animal models expressing PH-Tau, it was found that two putative mechanisms of neurodegeneration exist depending on the concentration of the toxic protein, both involving cognitive decline, due to synaptic dysfunction at lower concentration and neuronal death at higher. Studies investigating the mechanism of tau pathology and its transmission from neuron to neuron are currently ongoing.

Keywords: Hyperphosphorylation, microtubules, neurodegeneration, tau, tau mouse model

INTRODUCTION

Several dementias have in common the formation of intracellular filamentous deposits formed of the microtubule-associated protein tau, in abnormally hyperphosphorylated forms. Through abnormal tau function, they apparently share a common disease mechanism, and are collectively known as tauopathies. This family of diseases includes Alzheimer's disease (AD), frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), amyotrophic lateral sclerosis, cortical basal degeneration, dementia pugilistica, Pick's disease, progressive supranuclear palsy, and tangle-only dementia. Despite their diverse phenotypic manifestations, brain dysfunction, and degeneration, these tauopathies are linked to the progressive accumulation of filamentous hyperphosphorylated tau inclusions which, in the absence of other disease-specific neuropathological abnormalities, provide circumstantial evidence implicating abnormal tau in the onset and/or progression of neurodegenerative disease.

Our tau-research project started in Khalid and Inge Grundke-Iqbal's laboratory in 1992. The microtubule associated protein tau, originally described by the

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Kirschner laboratory in 1975 [1], was identified as the building block from the filamentous aggregates found in the neurons of AD patients. In the abnormal state, tau was hyperphosphorylated. Tau is normally found in cells as a phosphoprotein with ~3 moles of phosphate per mole of the normal protein. The hyperphosphorylated tau contains a significantly higher phosphate content than the normal tau, up to ~7–10 moles of phosphate per mole of protein [2] which includes the appearance of new phosphorylation sites. In the central nervous system, tau is a family of six proteins derived from a single gene by alternative splicing of the pro-mRNA [3, 4]. The human brain tau isoforms range from 352 to 441 amino acids, differing in whether they contain three or four tubulin-binding domains/repeats (R) which consist of 31 or 32 amino acids near the C-terminus. At the N-terminus of tau there are two, one, or no inserts of a 29-amino acid repeat (N). Isoform expression and degree of phosphorylation are developmentally regulated. Fetal tau is mainly composed of the 3R0N isoform and is highly phosphorylated normally, but lacks several of the phosphorylation sites seen in paired helical filament (PHF) tau. The degree of phosphorylation of the six isoforms decreases with age, probably because of the activation of phosphatases [5]. All six isoforms have been observed in hyperphosphorylated states in PHFs from AD patients [6–9].

Our interest was in the biological activity of tau as a microtubule associated protein (MAP). We found that hyperphosphorylated tau from AD brains was unable to promote microtubule assembly and that, more importantly, the abnormal protein could inhibit the normal activity of tau and other MAPs. AD P-tau was able to disrupt preformed microtubules and, for the first time, we described a prion-like behavior in tau: the abnormal protein was able to bind normal protein and turn it into an AD P-tau like molecule. We were the first to describe this gain of toxic function in tau in AD patients [10–13]. The toxic behavior was prevalent when AD P-tau was not forming filaments and it was lost upon dephosphorylation.

The majority of axonal proteins are synthesized in the neuronal cell body and transported through the axons along the microtubule tracks. Axonal transport occurs throughout the life of a neuron and is essential to its growth and survival. *In vitro*, tau promotes the assembly of tubulin into microtubules and stabilizes the assembled ones [1]. In the neurons of patients with AD, the microtubule system is disrupted, interrupting axonal transport, thus preventing vesicles from reaching the synapses. We have shown that hyperphosphorylated tau can disrupt these microtubules by sequestering normal tau through protein-protein interactions [10–13]. As a result, slowly and steadily, the synapses deteriorate by retrograde degeneration.

The discovery in 1998 of mutations in the tau gene, which co-segregate with the disease in frontotemporal dementia, provided unequivocal evidence that tau abnormalities alone are enough to cause neurodegenerative disease [14–16]. Three different types of tau mutations have been described: missense, intronic, and one-deletion (ΔLys280). The missense mutations resulted in point mutations that conferred disease progression (i.e., P301L and R406W). The intronic 5´ to exon 10 mutations resulted in overexpression of 4R tau proteins, disrupting the balance of 3R/4R proteins in neurons [15, 16]. The exact molecular mechanism of neurodegeneration in the affected patients is not yet understood. Like individuals with AD, FTDP-17 patients show accumulations of hyperphosphorylated tau as neurofibrillary tangles in every case. Hyperphosphorylated tau arises from the emergence of new phosphorylation sites in the protein. All the mutations discovered in tau are dominant, suggesting that the effect of tau mutations result in a gain of toxic function by the protein [17]. The research which was seeded in the Iqbal laboratory focused on understanding the mechanism of tau induced neurodegeneration by first understanding tau's biological activity as well as the biological activity of hyperphosphorylated tau, using biochemistry, cellular biology, and, most recently, animal models generated to express PH-Tau in neuronal cells. We proposed that mutations on tau associated with disease (FTDP-17) altered its conformation to make it a better substrate for kinases [18]. Using phosphomimetics, we found that the minimum phospho-sites necessary to acquire such a toxic behavior of tau were at 199, 212, 231, and 262, and tau pseudophosphorylated at those sites behaved as pathological tau [19]. Here is a brief tale of our work on tau.

MICROTUBULES AND TAU IN ALZHEIMER'S DISEASE

When the neurons of patients with AD are studied, a decrease in microtubules is observed with a concurrent increase in the concentration of tau [2]. Three different pools of tau can be observed from the brains of AD patients: AD tau, not hyperphosphorylated and most similar to normal tau; AD

P-tau, soluble hyperphosphorylated tau; and PHF-tau, insoluble and hyperphosphorylated tau. Levels of AD tau are decreased by about 60% compared to tau found in normal brain. AD P-tau, as well as normally phosphorylated tau, can be isolated from AD brain in solution [2]. Using brain extracts, we studied the biological activity of tau from AD brains to determine the microtubule-promoting activity in *in vitro* assembly assays [10]. We found that AD tau has normal microtubule-promoting activity; conversely AD P-tau did not promote microtubule assembly. Even more we found that AD P-tau inhibited the microtubule assembly promoted by normal tau, MAP1A, MAP1B, and MAP2 [20]. After treatment with phosphatases, the microtubule-promoting activity was recovered implicating phosphorylation of tau as the mechanism of microtubule disruption. Interestingly, AD P-tau preincubated with normal tau prior to the addition of tubulin both inhibited the normal microtubule–promoting activity *and* destroyed microtubules already present. This was probably due to interactions between tau and AD P-tau thereby sequestering it from the tubulin.

AD P-TAU HAS A PRION-LIKE BEHAVIOR

Using both solid phase and solution binding assays, we verified that AD P-tau was able to bind normal tau [11]. Quantitation of the solution binding assay indicated that the AD P-tau binding to normal tau was non-saturable, and visualization by electron microscopy showed us that the products were bundles of filaments [11]. These results suggested that hyperphosphorylation of tau could change the conformation of the protein in such a way that this change could be transferred to normal protein which would seed tau filament self-assembly. This was a new hypothesis in the field of tau biochemistry. The ability of hyperphosphorylated tau to bind normal tau was confirmed by Vandebroek et al. [21] in yeast. They expressed the human largest four-repeat protein (4R2N) and the human largest three-repeat isoform (3R2N), and demonstrated that human tau expressed in yeast acquired pathological phospho-epitopes, assumed a pathological conformation, and formed aggregates. These processes were modulated by yeast kinases Mds1 and Pho85, orthologues of GSK-3β and cdk5 which are kinases known to phosphorylate tau in humans (as reviewed in [22]). They observed that a) tau aggregated more when it was more phosphorylated, b) the mobility in SDS electrophoresis was higher with increased phosphorylation, c) isolated hyperphosphorylated tau was able to assemble into filaments, and d) the isolated hyperphosphorylated tau was able to nucleate the assembly of the normal, non-phosphorylated tau. The authors proposed that hyperphosphorylated tau is the biochemically stable form of tau that is the actual seed or nucleation factor that initiates and promotes the aggregation of tau, as we had proposed for hyperphosphorylated tau isolated from AD brain almost 10 years prior [11]!

The conformational change transfer by AD P-tau to normal tau is a property of a prion protein, and we were the first to describe this property in tau and to show that it was due to hyperphosphorylation. This prion-like activity of AD P-tau was further determined to disrupt the microtubules formed by normal tau or by the other neuronal MAPs, including MAP1b and MAP2 [11, 20]. Furthermore, amorphous aggregates are formed when AD P-tau binds to MAP1b and MAP2 [20].

TAU SELF-ASSEMBLY AND 'AD P-TAU-LIKE' PROTEIN BEHAVIOR IS INDUCED BY HYPERPHOSPHORYLATION

In AD, hyperphosphorylation of tau appears to precede the appearance of the tangles [8]. As described above, tau is a phosphoprotein that in its toxic, hyperphosphorylated state has an increase of 2–4 times the phosphate per mole of protein due to an increase in the number of phospho-sites [2]. Degenerating neurons appear to have tau that has self-assembled into tangles composed of PHFs and short filaments (SFs). AD P-tau was able to self-assemble into these tangles (Fig. 1A) at varying pHs [13]. The PHFs generated by AD P-tau *in vitro* had similar dimensions to those of AD PHFs extracted from the brain. These filaments all contained a wide part of ∼20 nm, which narrowed to ∼10 nm at every ∼80 nm. Within the bundles of PHFs, some 4-nm protofilaments and SFs of ∼15 nm, similar to the SFs in AD, were also observed. The self-assembly was halted by dephosphorylation of AD P-tau (Fig. 1A) [10] suggesting that hyperphosphorylation of tau is a requirement for its self-assembly into tangles of filaments of varying sizes.

To confirm the role of hyperphosphorylation in the conversion of normal tau into a toxic molecule that has aggregation propensities, the six isoforms of recombinant tau (r-tau) were individually treated

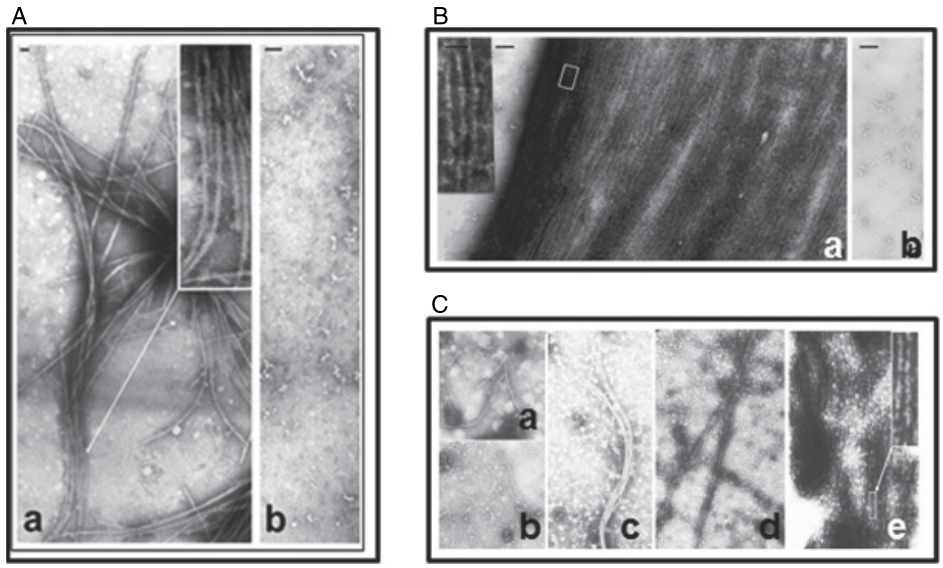

Fig. 1. *In vitro* polymerization of AD P-tau and recombinant tau into tangles of PHF/SF and the effects of dephosphorylation. A) AD P-tau was purified as described in Kopke et al. [2], 0.4 mg/mL (a) without pretreatment and (b) dephosphorylated by alkaline phosphatase was incubated for 90 min and the products of assembly were examined by negative stain electron microscopy. Dephosphorylation completely abolished AD P-tau polymerization. B) Recombinant tau, 0.5 mg/mL, was incubated with rat brain extract as a source of protein kinases in the presence of (a) ATP to induce hyperphosphorylation of tau or (b) non-hydrolyzable ATP, AMP-PNP as a contol. C) Recombinant 2N4R tau with FTDP-17 mutations, 0.5 mg/mL, were incubated with rat brain extract plus ATP to induce hyperphosphorylation then analyzed by negative stain electron microscopy (1-h incubation: a, V337M; b, R406W; 4-h incubation: c, R406W; 6-h incubation: d, P301L; G272V). The research for A and B was originally published in [13]; and the research for C was originally published in [18].

with protein kinases present in normal brain extract and followed its ability to bind normal tau and to inhibit its microtubule-promoting activity [12, 13]. Rat brain extract treated r-tau became hyperphosphorylated with the increase to ∼12 moles of phosphate per mole of the protein (phosphorylated tau, P-tau) which is similar to AD P-tau. P-tau also bound to normal tau and was able to self-assemble into tangles of PHFs/SFs in a phosphorylation dependent manner and inhibited the microtubule assembly activity (Fig. 1B) [13]. These results suggested that hyperphosphorylation could convert tau into an AD P-tau-like state.

Several reports have shown that FTDP-17 mutations decrease tau's ability to promote tubulin assembly into microtubules [23] or increase the ability of tau to self-assemble [24]. We proposed that these mutations may change the conformation of tau making it a better substrate for phosphorylation [25]. Phosphorylation assays using r-tau with FTDP-17 mutations R406W, P301L, V337M, or G272V resulted in faster rate and greater phosphorylation extent (∼16–18 moles versus ∼12 moles of phosphate per mole protein) than normal tau *in vitro* [18]. This increase in phosphorylation probably correlates to an increased number of sites that become modified based on the higher phosphorylation stoichiometry. We also found that fewer moles of phosphate per mole of protein were required for filament formation in the mutant proteins (Fig. 1C).

Upon excess phosphorylation, tau will acquire the ability to bind normal tau. This occurs maximally after the incorporation of ∼4 moles of phosphate per mole of protein [18] and polymerizes into filaments after ∼10 moles of phosphate per mole of protein [13, 18]. These results suggest that at least two different conformational states of tau are induced by phosphorylation: one in which the hyperphosphorylated tau is able to bind normal tau, and one in which it is able to self-assemble into filaments. These mechanisms may be regulated by changes in phosphorylation mediating the neutralization of the charged regions on the protein. As previously shown, the N-terminal inserts of tau can neutralize the positive charge of the flanking regions of tau and induce self-assembly of unmodified protein. In agreement with our model, oxidation of tau by the addition of carbonyls to Lys, which also neutralizes the charge, increases tau filament formation [26]. Different mechanisms can lead to the conformational change to acquire tau toxic conformation, that as we have shown, can be transferred to the normal, unmodified protein.

As discussed above, hyperphosphorylation confers upon tau a toxic property in which microtubule stability is decreased because of its ability to bind normal tau and MAPs. It is possible that this toxic property is lost upon increased self-assembly as PHF-Tau do not bind normal MAPs and do not inhibit microtubule assembly [27]. Our hypothesis is that tau gets hyperphosphorylated, binds normal MAPs, disrupts microtubules, and interrupts axoplasmic transport, with the consequent degeneration of the synapse. If tau self-assembles into PHF/SF, then it cannot bind normal MAPs, and the microtubules can be still functional.

WHAT IS HYPERPHOSPHORYLATED TAU? HOW CAN WE STUDY THE GAIN OF TOXIC FUNCTION?

Hyperphosphorylated tau is understood to be a protein in which there is an increase in moles of phosphate per mole of protein. However, there is much discussion as to whether hyperphosphorylation actually relates to a general increase in this ratio or increased phosphorylation at specific sites within the molecule. One method to mimic the negative charge of the phosphate group and length of the side chain is pseudophosphorylation where the codons for Ser or Thr residues are replaced with that for Glu. This is a widely accepted approach to mimic phosphorylation [19, 28–32]. A mouse model was developed to study hyperphosphorylated tau using a tau protein with 10 pseudophosphorylation sites [33]. This mouse did not appear to have any of the hallmark traits of dementia-related neurodegeneration indicating that it is more likely phosphorylation at specific sites than overall phosphate per molecule.

This understanding led us to more closely examine the role of protein conformation as the structure of tau, and other intrinsically disordered proteins, may be determined by long-range interactions which can be modulated by phosphorylation and other post-translational modifications [34]. Intermolecular association of tau has been linked to interactions through the microtubule binding domain (MTBD) while self-assembly appears to be inhibited by the flanking regions of this domain [18, 28, 35] (Fig. 2). The presence of the two N-terminal inserts of tau, which are highly negative, can induce tau self-assembly potentially by neutralizing the charge of the flanking region, as we have shown that non-modified full length tau is able to self-assemble in short filaments [13]. As a disordered protein, tau has little defined secondary structure. Nevertheless, the study of tau structure in PHF/SF from AD brains showed that the structure of tau is important in tau self-assembly [36], confirming our observations on tau self-assembly from the whole molecule. Regions of tau have a strong basic charge (pI > 9) and are separated from other domains by Pro residues, which can induce a bend in the amino acid chain. These very basic regions that are N-terminal to the microtubule binding domains can mask the intermolecular attraction of the MTBD. Three residues in this region, Thr212, Thr231, and Ser262, appear to be 50% phosphorylated when tau begins to polymerize [18] thus decreasing their theoretical pI and increasing the probability of tau self-assembly. On the C-terminal side of the MTBD there is a basic region up to Pro397 that is followed by an acidic segment. Phosphorylation at Ser396 and/or Ser404 may open up this segment and increase intermolecular interactions thereby increasing tau self-assembly.

Using this information, pseudophosphorylated sites were studied in the presence and absence of mutations related to FTDP-17, since it was shown to increase the phosphorylation effect. To determine which residues to change to Glu, recombinant tau was phosphorylated *in vitro* and the phosphorylated sites were determined at the point that self-assembly occurred, about 5 moles of phosphate incorporated per mole of protein by about 2 hours of incubation. Upon analysis, nine sites were found to be phosphorylated about 50%: Ser199, Ser202, Ser205, Thr212, Thr231, Ser235, Ser262, Ser396, and Ser404. From these results, we studied the tau gene (*MAPT*) mutated at each site to Ala (non-phosphorylatable) or Glu (pseudophosphorylated) in the normal tau or R406W background. Upon transfection into PC-12 cells, the vectors containing Ala mutations acted similarly to non-mutated tau at each of the sites tested. Mutations to Glu, in most cases, resulted in tau dissociation from tubulin but complete microtubule disruption was not observed [19]. This indicated to us that a single phosphorylation event was not enough to convert tau into an AD P-tau like toxic molecule.

After multiple combinations containing two or three pseudophosphorylation sites, it was determined that the strongest effect was observed with the triple mutant tauT212E/S235E/S262E which bound weakly to microtubules in CHO cells and decreased tubulin staining. This pseudophosphorylated tau appeared to be aggregated in both the cytoplasm and nuclear space and was able to sequester normal tau

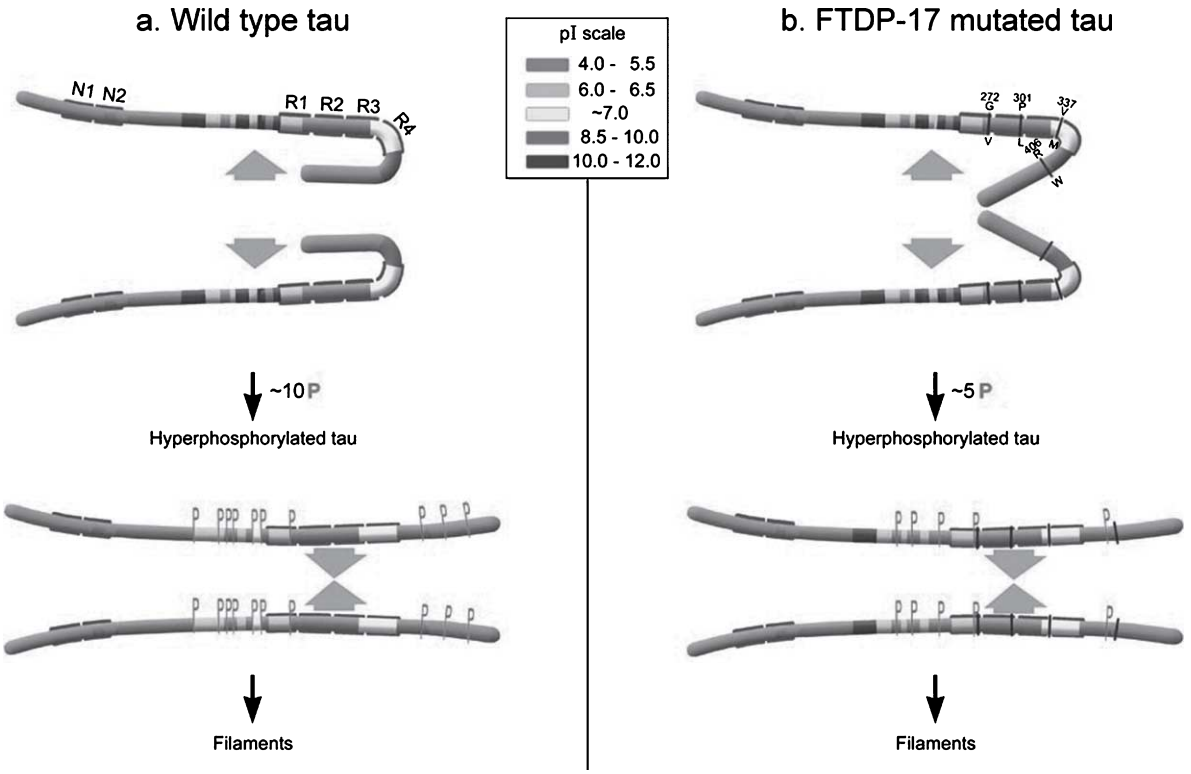

Fig. 2. A hypothetical scheme of the phosphorylation-induced self-assembly of wild-type and FTDP-17 mutated tau proteins. Tau self-assembles mainly through the microtubule binding domain/repeat R3 in 3R tau proteins and through R3 and R2 in 4R tau proteins (R2 and R3 have β-structure). Regions of tau molecule both N-terminal and C-terminal to the repeats are inhibitory. Hyperphosphorylation of tau neutralizes these basic inhibitory domains, enabling tau-tau interaction. In the case of the C-terminal region beyond Pro397 (398–441), a highly acidic segment masks the repeats. Phosphorylation (*red P*s) of tau at Ser396 and/or 404 opens this segment, allowing tau-tau interaction through the repeats. FTDP-17 mutations make tau a more favorable substrate for phosphorylation than the wild-type tau. The mutated tau proteins achieve the conformation required to self-assemble at a lower level of incorporated phosphate. Although the FTDP-17 mutant tau proteins have conformations that are more prone to polymerize, in the absence of hyperphosphorylation, the highly basic segments and the C-terminus interfere with polymerization. Phosphorylation sites are indicated by *red P*s at Ser/Thr positions in tau (left panel): 199, 202, 205, 212, 231, 235, 262, 396, 404, and 422; and in FTDP-17 mutant tau (right panel): 199, 212, 231, 262, and 396, respectively. This figure was reproduced with permission from *Alzheimer's & Dementia* [22].

in a manner similar to that of tau isolated from AD brain [19]. When compared to wildtype tau, we found that Ser199 in the pseudophosphorylated tau was very highly phosphorylated. This suggests that phosphorylation at these four sites is able to convert tau into a toxic species which was enhanced by the FTDP-17 mutation R406W. We decided that tau hyperphosphorylation was due to phosphorylation at specific sites within the molecule and we generated phospho-sites at these four residues with the R406W mutation and we named it Pathological Human Tau (PH-Tau).

TOXIC GAIN OF FUNCTION OBSERVED IN A TAUOPATHY MODELS

We generated tau-transgenic flies to study PH-Tau effects *in vivo*. We found in *Drosophilia* that PH-Tau expressed in a pan-neuronal fashion has a marked effect on the olfactory learning [37]. We have recently developed and characterized a new mouse model in which PH-Tau is expressed in neuronal cells under the control of the CaMKII promoter [38]. This model expressed the protein at two different levels: PH-Tau_{low} (4% of normal tau when the promoter is repressed) and PH-Tau_{high} (14% of normal tau when the promoter is induced). These levels may be correlated with the different levels of *in vitro* phosphorylation that change the tau binding abilities described above. Substantial differences in cognitive abilities, synaptic morphology, and neuronal loss were observed between PH-Tau_{low} and PH-Tau_{high} [38]. Low levels of PH-Tau resulted in cognitive deficits and reduced CA1 synapse number, synaptic protein levels were reduced and PH-Tau appeared

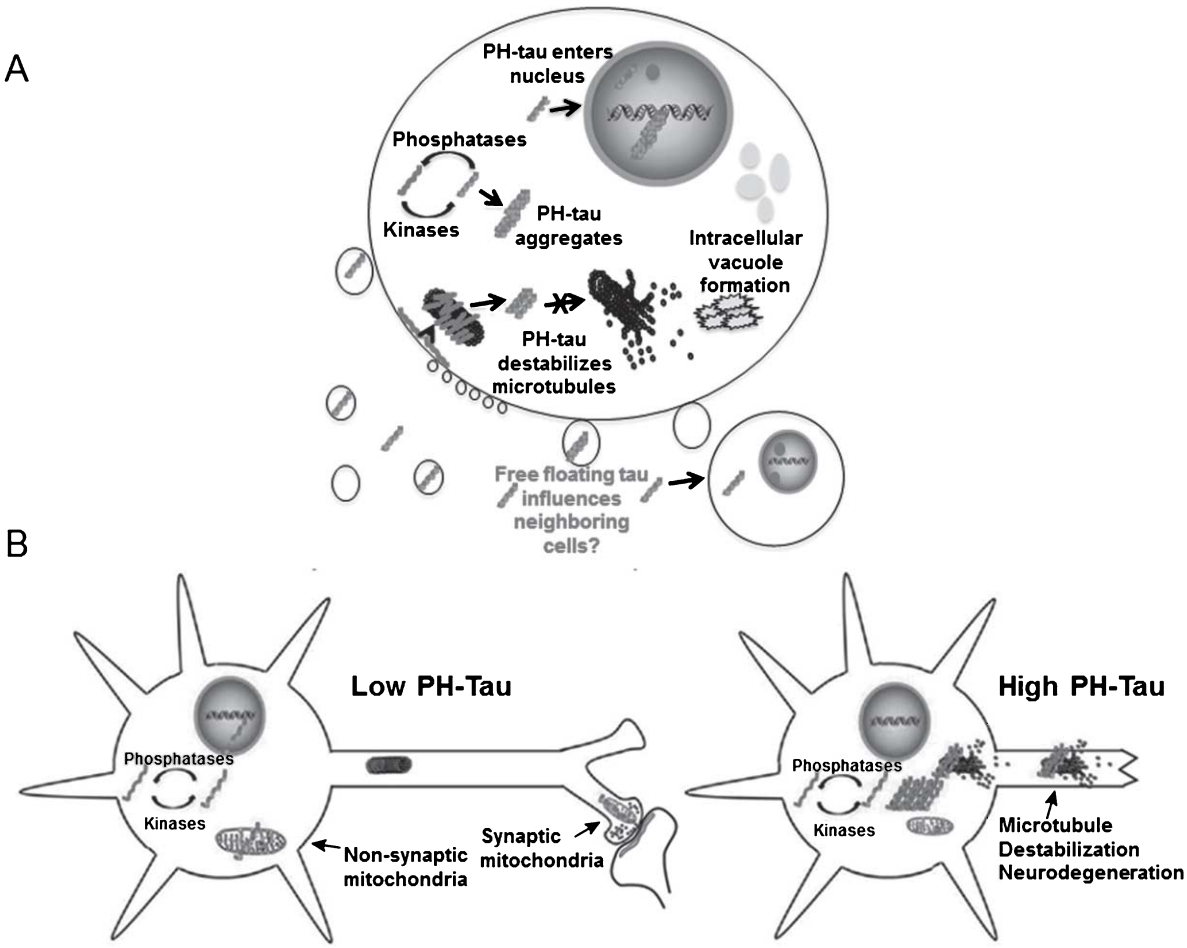

Fig. 3. Proposed mechanisms of neurodegeneration. A) PH-tau induces not only microtubule disruption, but it is also translocated in the nucleus, causes intracellular degeneration, protein aggregation, and vacuole formation. The presence of tau in the nucleus might be involved in alterations of protein expression. As a result of cell death or cell altered metabolism tau can be released from the cells, it is possible that the released conformationally altered tau molecule can propagate the disease to neighboring cells. This figure was reproduced with permission from *Alzhiemer's & Dementia* [22]. B) (Left) Low level of PH-tau expression results in translocation to the nucleus, synaptic dysfunction, and mitochondrial disruption. The presence of tau in the nucleus might be involved in alterations of protein expression. B) (Right) High levels of PH-Tau expression results in protein aggregation, microtubule disruption, and loss of synapses. As a result of cell death or cell altered metabolism tau can be released from the cells, it is possible that the released conformationally altered tau molecule can propagate the disease to neighboring cells. This figure was reproduced with permission from the editors of *Protein Folding Disorders in the Central Nervous System* [50].

to be present in the neuronal body and nuclei. With high levels of PH-Tau there was neuronal death primarily in CA3 as well as astrocytosis in certain brain regions with no apparent effect on CA1 synapses and the processes of the neurons had disappeared [38]. Interestingly, PH-Tau had distinct biochemical properties when expressed at low and high levels that could account for the different phenotypes. At low PH-Tau, we observed a high molecular weight tau species (∼100 kD) that was significantly reduced when high levels of PH-Tau were induced. Furthermore, PH-Tau in the induced animals was truncated at 421. Preliminary work in this mouse model indicates disruptions in mitochondrial morphology in the CA1 and CA3 regions of the hippocampus of mice expressing PH-Tau. These changes may be due to mitochondrial dysfunction that has been shown to play an increasing role in AD [39–46].

CONCLUSIONS AND VISIONS

Twenty years ago, our work showed that hyperphosphorylated tau sequesters healthy tau protein and causes healthy tau to become pathological though

the mechanism of neuronal death was unclear [11]. Through the years, and through much hard work by many researchers in the field, a clearer picture is being drawn. Our studies, including biochemical data using tau from AD brains and recombinant tau as well as the studies using pseudophosphorylated tau, allow us to better understand the modifications of tau that can modulate different events at the cellular levels with important consequences for its physiology (Fig. 3). We have observed tau translocation into the cell nucleus [19]. Presence in the nucleus can cause hyperphosphorylated tau to alter the interaction with DNA [47] and may influence protein expression, in turn affecting cellular function. It is known that hyperphosphorylated tau, especially when it has other mutations, causes not only a destabilization of the microtubules (see above), but also the actin microfilaments [48]. Disruption of the microfilaments in cells can lead to zeiosis of the cell membrane. We have observed in cell culture that as the membrane pinches off during exocytosis, there is the release of hyperphosphorylated tau-containing membrane vesicles throughout the surrounding cellular environment (data not shown). We propose that these vesicles drift toward, and interact with, surrounding cells and that the contents are taken up by endocytosis. As the pathological protein moves from cell to cell it can sequester more healthy tau, propagating its prion-like behavior from neuron to neuron, causing a disruption of all cytoskeleton components, destabilizing the organelles, disrupting protein synthesis, and eventually inducing zeiosis and continuing disease transmission (Fig. 3A).

We could picture different scenarios where the levels of hyperphosphorylated tau start appearing in the cell because of kinase overactivity, phosphatase deficiency, changes in the substrate conformation, failure in the clearance system, or a combination of them. At the beginning of the diseases, the conformationally modified tau might move in the cell, translocating in the nucleus, locating in synapses, interfering with mitochondria homeostasis (Fig. 3B left). As a consequence, cognitive impairment without significant structural changes might be observed [38]. As the pathological tau increases in the neurons, the toxic effect on the cytoskeleton and the retrograde neurodegeneration appears (Fig. 3B right). Our results reinforce the key role of tau in the development of pathology, in AD and other tauopathies. Despite the different mechanisms, it appears that reduction in the levels of hyperphosphorylated tau remains a key target for tauopathies, in combination with therapies to prevent cytoskeleton disruption [49]. Understanding the mechanism of transmission will allow us to design blockers of tau secretion and/or uptake, or regulators of microglia or other mechanisms to reduce extracellular pathological tau, which will help us halt the progression of the disease. From our models, it is apparent that low levels of conformationally altered tau is enough to trigger pathological effects. To understand these mechanisms triggered by tau but seemingly unrelated to microtubule structure will point to new therapeutic target development.

ACKNOWLEDGMENTS

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Amyloid-β and Tau in Alzheimer's Disease: Novel Pathomechanisms and Non-Pharmacological Treatment Strategies

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Abstract. Accumulation of the peptide amyloid-β (Aβ) and the protein tau in Alzheimer's disease (AD) brains is a gradual process that involves the post-translational modification and assembly of monomeric forms into larger structures that eventually form fibrillar inclusions. This process is thought to both drive and initiate AD. However, why the axonally enriched tau in the course of AD accumulates in the somatodendritic domain is not fully understood. We discuss new data that provide a possible explanation that involves *de novo* protein synthesis, induced by Aβ and mediated through the kinase Fyn. We further discuss how in a pathological state, tau, being a scaffolding protein, impairs nuclear and mitochondrial functions and reduces action potential generation at the axon initial segment. Pathological tau can further be packaged into exosomes, released by one neuron and taken up by another, contributing to its pathogenicity. We also present our new work that suggests ultrasound as a new treatment modality to clear pathological Aβ and tau. We put this work into perspective, discussing current vaccination strategies and improved brain delivery methods involving antibody engineering and viral approaches. We propose that rather than reducing post-translational modifications of tau, its levels and *de novo* synthesis need to be reduced. We anticipate a surge in combinatorial strategies, simultaneously targeting multiple pathologies, and an improved drug delivery to the brain facilitated by emerging technologies such as ultrasound.

Keywords: Alzheimer's disease, amyloid, axon initial segment, focused ultrasound, fyn kinase, microtubule-associated protein tau, non-invasive, phosphorylation, spreading, vaccination

INTRODUCTION

In this article, we are focusing on the last five years of work originating from our laboratory. Our research is based on the assumption, that amyloid-β (Aβ) and tau, the major proteinaceous constituents of the two hallmark lesions of Alzheimer's disease (AD), the amyloid plaques and the neurofibrillary tangles, not only constitute biomarkers, but in fact initiate and drive the disease process, presenting these molecules as appropriate targets for therapeutic intervention. We discuss our recent mechanistic work that contributes to a better understanding of how Aβ 'talks' to tau and how tau causes neurodegeneration. This includes a new mechanism of how neuronal tau accumulates in the somatodendritic domain as the disease unfolds. For much of our work we rely on

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transgenic mouse models with either Aβ or tau deposition. We also used these mice to develop a novel therapeutic approach termed 'scanning ultrasound' (SUS) that builds on ultrasound research going back several decades, and demonstrated that both Aβ and tau aggregates can be effectively cleared with ultrasound, restoring neuronal functions. Regarding tau, we combined SUS with a therapeutic anti-tau antibody fragment to achieve synergistic therapeutic effects. We will put our work into perspective by comparing it to ongoing treatment strategies, in particular vaccinations. Finally, we will discuss how we believe the new pathomechanistic insight will influence therapeutic strategies. We anticipate that combinatorial approaches will lead to better therapeutic outcomes. In this context, 'combinatorial' not only refers to the simultaneous targeting of two pathologies (such as Aβ and tau), but also to the combination of a drug with a non-pharmacological procedure.

PATHOMECHANISMS OF TAU

Tau belongs to a family of microtubule-associated proteins that also includes MAP2 and MAP4. These proteins share repeat motifs with which they bind to microtubules. AD brains are histopathologically defined by extracellular amyloid plaques containing the peptide Aβ and intracellular neurofibrillary tangles containing the microtubule-associated protein tau. Aβ is derived by proteolytic cleavage from the larger amyloid-β protein precursor, AβPP. In AD, Aβ is thought to accumulate both because of an increased production and an impaired clearance [1]. The process toward plaque formation involves oligomerization and fibrillization of Aβ. A similar process is known to occur for tau that becomes hyperphosphorylated (p-tau) before forming fibrillar aggregates [2]. Tau pathology in the absence of Aβ deposition is prevalent in several other diseases that are collectively termed tauopathies and includes frontotemporal lobar degeneration (FTLD).

A crucial role for p-tau in the neurotoxicity and degeneration observed in AD and related tauopathies has been demonstrated by us and others, in part by using transgenic mouse models that, in an age-dependent manner, recapitulate major aspects of the human pathology [3]. A role for aging was also demonstrated when pR5 mice expressing P301L mutant human tau found in familial FTLD were back-crossed onto a senescence-accelerated SAMP8 background. We found that this exacerbated the pre-existing pathology that characterizes the tau transgenic mice, presenting this novel strain as a tool to screen for disease-modifying factors [4]. Within the limitation of a mouse model, strains such as Tau58-2/B that express the P301S mutation of tau also recapitulate neurological deficits of distinct tauopathies, such as the behavioral variant of frontotemporal dementia. By assessing Tau58-2/B mice in a comprehensive behavioral test battery, we found that the tauopathy mice showed age-dependent signs of impulsivity and decreased social exploration and executive dysfunction. The deficit in executive function was first limited to decreased spatial working memory, but with aging this was extended to impaired instrumental short-term memory, presenting the mice as a suitable model to test therapeutic interventions for the amelioration of this tauopathy variant [5].

An interesting observation can be made when one addresses the fate of distinct p-tau epitopes in mice. We had previously found in the pR5 mice, that whereas pathological p-tau epitopes such as AT180 (T231) or AT270 (T175/T181) become increasingly phosphorylated in vulnerable areas such as the hippocampus and the amygdala as the disease progresses, the AT8 epitope (S202/T205) goes through a biphasic stage: For example, in the CA1 region, at 3 months, AT8 staining is faint. Once the mice reach 6 months, AT8 staining intensity increases. At 20 months, a remarkable change in the AT8 pattern becomes evident, as staining is now being confined to just a few neurons with rich arborization, and this staining is very intense [6]. Taking this further, in collaborative work, we found that in this second phase, when these neurons undergo intense, fibrillar changes, phosphorylation of additional pathological serine/threonine epitopes such as AT100 (T212/S214) was also massively increased [7]. This was associated with an activation of the tyrosine kinase Pyk2 (also known as Ptk2b) (that has since been identified as an AD risk gene [8]) as well as its putative substrate GSK3β via tyrosine phosphorylation, which may explain the massive pathological phosphorylation of tau in this second stage [7].

Tau transgenic mice were useful in identifying pathomechanisms that affect a range of cellular functions which is not surprising considering that tau is a scaffolding protein interacting with many proteins in an isoform-dependent manner [9]. Multiple aspects of mitochondrial function are impaired by pathological tau [10], as we already showed in 2005 for the oxidative phosphorylation system [11]. More recent collaborative work revealed a major

role for tau in impairing mitochondrial fission by preventing the efficient recruitment of Drp1 onto mitochondria, leading to elongated mitochondria [12, 13]. The pR5 mice were also useful for identifying another pathomechanism that involves nuclear depletion and cytoplasmic accumulation of the nuclear factor SFPQ (also known as PSF) [14]. Interestingly, loss of SFPQ function has since been shown to alter the tau isoform ratio and cause an FTLD-like phenotype [15].

Tau in also known to impair synaptic activity as has been shown in numerous studies; however, tau's impact on neuronal excitability has received remarkably little attention, although it has been reported that the removal of tau reduces network hyperexcitability [16]. In very recent work, we have shown that p-tau induces a more depolarized threshold for action potential initiation and reduces firing in hippocampal CA1 neurons of tau transgenic mice, which was rescued by the suppression of transgenic tau. Furthermore, in primary hippocampal neuronal cultures, we revealed that this reduction in neuronal excitability resulted from the relocation of the axon initial segment (AIS) down the axon in a tau phosphorylation-dependent manner. This suggests that a reduction in hippocampal excitability due to a tau-mediated distal re-localization of the AIS contributes to the hippocampal dysfunction observed in tauopathies [17].

An interesting new thread has been added to the field with the notion that tau pathology propagates extracellularly [18, 19]. This notion has its foundation in the distribution of neurofibrillary tangles that follow a distinct pattern through anatomically connected brain regions and the well documented correlation between the severity of tau pathology and the disease progression implies a 'prion-like' seeding and spreading mechanism of p-tau [20]. One mechanism by which p-tau can spread is through being packaged into extracellular vesicles (EVs), membranous vesicles 30–1,000 nm in diameter. We have demonstrated *in vitro* that p-tau is contained within EVs enriched for exosomes isolated from either wild-type mice or rTg4510 mice with a pronounced tau pathology and have shown that the tau within EVs is able to seed the aggregation of endogenous tau in recipient cells in a threshold-dependent manner [21]. Furthermore, we have shown *in vivo* that transgenic EVs cause increased tau phosphorylation and soluble oligomer formation in a manner comparable to that of freely available proteins in brain lysates in human tau transgenic ALZ17 mice [22]. Another approach to address tau spreading pursued by us was by assessing the spreading of endogenous phosphorylated tau. To generate endogenous seeds, we injected the protein phosphatase 2A (PP2A) inhibitor okadaic acid (OA) unilaterally into the amygdala of wild-type mice and found that this insult rapidly initiated changes in tau phosphorylation, solubility, and aggregation at anatomically distant sites. More specifically, we detected protein aggregation via thioflavin-S at the injection site and in the cortex of both injected and contralateral hemispheres, which was not induced in tau knock-out mice. Together, this suggested to us that tau phosphorylation can be both a primary response to an insult, and a secondary response communicated to non-exposed brains regions [23]. Taken together this and the work of others demonstrates that extracellular vesicles can transmit tau pathology, indicating a role for extracellular vesicles in the transmission and spreading of tau pathology.

HOW Aβ DRIVES TAU PATHOLOGY: A CENTRAL ROLE FOR THE KINASE FYN

Synaptic degeneration precedes neuronal loss in AD, and not surprisingly, AD has been termed a synaptic failure [24]. Furthermore, Aβ is believed to drive tau pathology which presents the challenging question as to how these molecules actually interact, considering that Aβ is released into the extracellular space, whereas tau that early in development is distributed throughout the neuron, becomes enriched in the axon with neuronal maturation [25]. In addressing this question, we have shown previously that tau is also found in dendrites, albeit at lower levels as in the axon, where it is required to target the kinase Fyn to the spines, mediating Aβ toxicity [26]. More specifically, we found that Fyn phosphorylates the NMDAR subunit NR2b which facilitates the recruitment of PSD95 to form an excitotoxic complex through which Aβ exerts its toxicity. Others have also contributed to this concept [27–29]. Interestingly, distinct forms of Aβ lead to specific phosphorylation events as shown in a collaborative effort for Aβ*56 that activates CaMLIIα which is associated with increased site-specific phosphorylation (S202, S416) and missorting of tau [30]. Localization of tau itself to dendritic spines is phosphorylation-dependent as has been shown by expressing pseudophosphorylated forms of tau [31, 32]. We have also used the genome-editing tool TALEN to generate Tau-mEOS2 knock-in mice which showed that Tau-mEOS2 fol-

lowed a proximo-distal gradient in axons and a subcellular distribution similar to that of endogenous Tau in neurons obtained from wild-type mice. This was abolished, when either hWT-Tau or hP301L-Tau was overexpressed—a situation resembling that in disease where tau levels are also increased and distort the physiological distribution of tau [33].

It is generally assumed that hyperphosphorylated tau in the axon detaches from the microtubules and passes through the AIS, which serves as a diffusion barrier for physiologically phosphorylated tau, before accumulating in the cell body and dendrites, a process that is partly mediated by Aβ [34, 35]. However, again the question of compartmentalization arises and we asked ourselves whether Aβ may employ a mechanism other than relocalization of tau to account for the massive accumulation of tau in the somatodendritic compartment. Indeed, we identified an additional, and as we believe, more cogent mechanism that involves local Aβ-mediated protein translation of tau in the somatodendritic domain [36]. More specifically, we found that this activation occurred through a signaling cascade that involves Fyn, the serine/threonine-directed kinase ERK as well as the ribosomal protein S6, and the activation of this cascade is associated with an increased phosphorylation of tau at multiple residues. Together, these findings reveal *de novo* protein synthesis of tau in the somatodendritic compartment, mediated by Aβ, as a novel pathomechanism in AD.

Aβ CLEARANCE AND MEMORY RESTORATION: ESTABLISHING SCANNING ULTRASOUND (SUS) AS A NON-PHARMACOLOGICAL AND NON-INVASIVE THERAPEUTIC STRATEGY

The blood-brain barrier (BBB) is a selective structure that protects the brain parenchyma from circulating factors and restricts access of pathogens and immune cells to the brain [37]. However, this also means that the BBB presents a significant challenge for AD therapeutics, as the vast majority of potentially effective drugs are blocked from accessing the brain and engaging with target molecules in the brain. Repeated high doses of therapeutic molecules are currently required to achieve efficacy following systemic injection in animal studies which poses a significant challenge for translation into humans. This highlights the need for better delivery strategies to reduce both the cost and dose of treatment [38]. One potential way to achieve this goal is the use of ultrasound to transiently open the BBB to access the brain, a method that has been explored by us.

Ultrasound is a type of mechanical energy that is defined as the acoustic wave propagation in a medium at frequencies exceeding the range of human hearing, i.e., above 20 kHz. Different from visual light and other electromagnetic waves such as radio waves, microwaves, or x-rays, acoustic waves can penetrate solids and liquids and bounce back from impediments or when encountering abrupt changes. This explains their suitability for imaging light-impenetrable objects non-destructively. Because of the inherent diffraction limit of the resolution for any kind of wave [39], sound in the normal human hearing range, i.e., with a wavelength above 10 cm, can only resolve large objects. To obtain a higher resolution, a higher acoustic frequency is needed, as is the case for ultrasound that in the medical space is routinely used as an imaging modality for diagnostic applications, primarily in the fields of obstetrics and cardiology, but also for examining the abdomen and musculoskeletal system. In this situation, ultrasound waves are transmitted from the transducer into the patient and then received as echoes by the same transducer, as the wave is partially reflected at tissue interfaces. Important for what we are reviewing here, ultrasound has been explored in recent years as a treatment modality for brain diseases [40].

In order to manipulate the BBB for targeted drug or gene delivery, non-thermal ultrasound can be used to capitalize on the interaction between ultrasound and microscopic bubbles of gas (microbubbles) in tissue or fluids [41]. Microbubbles might pre-exist in tissue, but damaging acoustic pressure is required to generate the necessary cavitating microbubbles [42]. Therefore, preformed, commercially available microbubbles are being used to ensure biological effects even at low acoustic pressures [43]. These microbubbles are routinely used for contrast-enhanced ultrasound imaging. They are biologically inert and have a gas core encapsulated by a thin shell of lipid or polymer. Ultrasound causes them to cavitate, i.e., to expand and contract, resulting in vessel wall displacement [44–46], a process termed by us 'obicodilation'. Displacement causes a transient opening of endothelial tight junctions because of the disintegration of the associated junction complexes. This transiently facilitates transport across the BBB [47].

Several studies have examined obicodilation as a means to specifically target AD pathology. In one such study, an Aβ-specific antibody was shown to reduce Aβ pathology in TgCRND8 mice, coupled with magnetic resonance imaging (MRI) monitoring using the contrast agent gadobutrol. Application of MRI-guided focused ultrasound to four locations in the right hemisphere reduced Aβ pathology relative to the corresponding areas in the untreated contralateral hemisphere [48]. Neither injection of the antibody nor sonication alone was effective. In a follow-up study, Aβ pathology was reduced even in the absence of a therapeutic antibody [49]. The effect was, however, very modest, suggesting that obicodilation is best used as a delivery tool for peripherally administered anti-Aβ antibodies [49]. Nevertheless, in the absence of an antibody, bilateral sonication of the hippocampus in TgCRND8 mice once per week for 1 month led to a 20% reduction in plaques, restored spatial working memory, and increased hippocampal neurogenesis [50].

An alternative to targeting a small, defined area with ultrasound is to move the ultrasound beam stepwise over the entire skull, thereby opening the BBB throughout the brain, an approach developed by us and termed scanning ultrasound (SUS) [51]. We applied this strategy to two large cohorts of Aβ-depositing and cognitively impaired APP23 mice [26], in the absence of any therapeutic agent. The mice were sonicated in the presence of microbubbles (i.e., obicodilated) once per week for a total of 6–9 weeks. This resulted in a two-fold reduction in plaque burden, and an up to five-fold decrease in monomeric and oligomeric Aβ species. Of note, this reduction is comparable to what is routinely achieved by Aβ-targeted vaccination trials. We also performed an extensive safety study that suggested to us that SUS is a safe method to transiently open the BBB. There were neither 'dark' neurons as revealed by Nissl staining, nor edemas or erythrocyte extravasation as shown by hematoxylin and eosin staining. Using the acid fuchsin stain, we found no evidence for ischemic damage. We further investigated the nuclear localization of NFkB, a marker of excessive, chronic inflammation, and the astrocytic marker GFAP and again, found no adverse effect of SUS treatment. In fact, this adds to the extensive safety literature that is already available for many species up to even macaques [40]. Safety has also been demonstrated in a recent clinical trial that used obicodilation to deliver a chemotherapeutic antibody to brain tumors [52]. Importantly, SUS treatment of APP23 mice not only reduced the Aβ pathology significantly, but also restored memory functions to wild-type levels, as shown with three complementary tests. As an underlying clearance mechanism, activation of microglia and uptake of Aβ into their lysosomes was identified, possibly mediated by blood-borne factors that entered the brain through the BBB and stimulated the dormant microglia [51].

In order to obtain additional insight into safety, we performed patch-clamp recordings from hippocampal CA1 pyramidal neurons in wild-type mice 2 and 24 hours after a single SUS treatment, and one week and 3 months after six weekly SUS treatments, including sham treatments as controls. Mice that received multiple SUS/sham treatments were, after aging for one week or 3 months following the final treatment, 6 and 9 months old, respectively, when the electrophysiological recordings and dendritic analysis were performed. In both treatment regimes, no changes in CA1 neuronal excitability were observed in SUS-treated neurons when compared to sham-treated neurons at any time-point. For the multiple treatment groups, we also determined the dendritic morphology and spine densities of the neurons from which we had recorded. The apical trees of sham-treated neurons were reduced at the 3-month time-point when compared to one week; however, surprisingly, no longitudinal change was detected in the apical dendritic trees of SUS-treated neurons. In contrast, the length and complexity of the basal dendritic trees were not affected by SUS treatment at either time-point. The apical dendritic spine densities were reduced, independent of the treatment group, at 3 months compared to one week. Collectively, these data suggest that ultrasound can be employed to prevent an age-associated loss of dendritic structure without impairing neuronal excitability [53]. What has not been determined is how SUS affects dendritic morphology in old mice and whether behavioral readouts will be affected, and more generally, whether ultrasound could be used as a cognition enhancement tool in healthy people.

TAU CLEARANCE AND BEHAVIORAL IMPROVEMENT: ENHANCING VACCINATION STRATEGIES BY USING ULTRASOUND AS A DELIVERY TOOL

With the exhaustive evidence that now supports a critical role for pathological tau in AD and related tauopathies and considering the recent failure of

many anti-Aβ therapeutics in clinical trials, therapies targeting tau have been rapidly increasing [54]. To promote the clearance of p-tau, we and others have generated antibodies specific for phosphorylated tau epitopes shown to be elevated in AD [55–59].

While passive immunization with the majority of these antibodies has demonstrated a reduction in tau pathology, these have been modest and behavioral improvements have only been achieved in some instances [58, 60]. For example, we have previously targeted the PHF1 (S396/S404) p-tau epitope, by both active and passive vaccination [61, 62]. This revealed some efficacy as determined for biochemical and histological read-outs, but no improvement in behavioral readouts. Rather than targeting pathological epitopes, reducing total tau levels therefore may be more beneficial especially as multiple studies have demonstrated that genetic ablation of endogenous tau does not cause behavioral or neuroanatomical abnormalities [26, 63]. This suggested that therapeutics designed to reduce total tau will be well tolerated. Recently, several groups have employed tau-lowering strategies at the mRNA and protein level to reduce total tau levels in the neuron, thereby reducing the progression of the disease. Antisense oligonucleotides (ASOs) were shown to successfully reduce tau expression in the PS19 tauopathy mouse model resulting in a significant decrease and even reversal of p-tau pathology as well as inhibition of hippocampal and neuronal loss, reversed tau seeding and reduced deficit in survival and nesting behavior [64]. Furthermore, this study provided additional evidence in nonhuman primates, that ASOs targeting monkey tau were highly efficacious at reducing endogenous tau mRNA and protein throughout the brain, spinal cord, and cerebrospinal fluid.

Pan-tau antibodies, on the other hand, can effectively reduce tau at the protein level. Intravenous injection with antibody 43D to the amino-terminal domain of tau (tau epitope 6–18) not only reduced tau pathology, but also Aβ pathology in the 3xTg AD mouse model, demonstrating for the first time that a tau therapeutic can also promote the clearance of Aβ. When compared to another anti-tau antibody, 77E9, specific for tau 184–195, 43D proved more effective at reducing tau pathology, rescuing cognitive deficits and ameliorating Aβ pathology [65]. This was also demonstrated with the anti-tau antibodies HJ8.5 (tau 25–30), HJ9.4 (tau 7–13), and HJ9.3 (tau 306–320) which were all demonstrated to block tau seeding *in vitro*, but only HJ8.5 and HJ9.4 rescued contextual fear deficits in mice [66]. Furthermore, peripheral administration of HJ8.5 to human patients with tauopathies and to human tau transgenic mice increased plasma tau levels in a dose-dependent manner [67]. Reduced tau uptake was also observed in an epitope-dependent manner with anti-tau antibodies Tau13 (N-terminal), 6C5 and HT7 (mid-domain) and Tau46 (C-terminal), whereby the N-terminal and mid-domain antibodies successfully prevented the uptake of tau species whereas the distal C-terminal specific antibody had little effect [68]. It is therefore believed that the site targeted by tau antibodies, rather than affinity, is important for reducing pathological tau *in vivo* and that targeting the N-terminus is expected to have the greatest effect. Taken together, the results from these recent studies present total tau as an alternative target to pathological tau for the treatment of AD and related tauopathies (see our recent review: [69]).

The mechanism by which antibodies reduce tau levels is still unclear, however, as the vast majority of anti-tau antibodies have not been detected intraneuronally, it has been suggested that they may engage extracellular tau and prevent tau seeding and spreading. To investigate whether antibody-mediated microglial activation and subsequent phagocytosis of the tau-antibody complex is required to reduce tau pathology, studies have investigated anti-tau antibodies with mutant Fc regions or antibodies which completely lack the Fc region all together. In a study which compared a full-effector version of an anti-tau antibody to an effector-less version, generated by mutating the Fc region of the antibody, it was found that the effector function is not required for efficacy *in vivo* and that, although full-effector function anti-tau promotes microglial uptake of extracellular tau, it also elicits microglial release of pro-inflammatory cytokines which is potentially deleterious to neurons [70]. This suggests that effector-less antibodies may not only be a more effective approach for targeting tau, but also a safer one.

An alternative approach to rendering antibodies effector-less is to remove the Fc region altogether through the generation of either fragment antigen binding (Fab) or single chain fragment variable (scFv) antibodies. This also reduces the size of the antibody, increasing tissue penetration and may therefore facilitate transfer across the BBB and neuronal membranes, allowing intraneuronal targeting of tau. As tau is predominantly localized within neurons this may achieve greater therapeutic outcomes. We recently explored the ability of an anti-tau scFv

in reducing pathological tau in the pR5 tau transgenic mouse model [71]. In our choice of antibody specificity, we were guided by earlier work from our team that had indicated that the 2N isoform of tau is particularly linked to disease [9, 72]. We isolated a 2N tau specific full-length antibody with a high affinity and specificity for 2N tau and converted it into an scFv format. We designed a preclinical study with four treatment arms and firstly found, that a tau isoform-specific scFv, RN2N, which binds to amino acids 84–97 of full length tau, was capable of inhibiting p-tau formation at N-terminal epitopes, thereby reducing overall pathological tau levels and improving behavioural outcomes after passive immunization in the pR5 mice. In the study we provided evidence that the antibody fragment or scFv prevents GSK3β-mediated phosphorylation of epitopes in the N-terminal half of tau. In agreement with our study, Ising and colleagues treated P301S transgenic mice with the HJ8.5 scFv and achieved a marked decrease of p-tau accumulation in the hippocampus of the mice by preventing the seeding of extracellular tau [73]. This demonstrates that antibody fragment binding of tau is sufficient to prevent it from undergoing hyperphosphorylation, aggregation and spreading, without the additional requirement for effector function. This is particularly advantageous in terms of safety as effector-less antibodies overcome a potentially dangerous inflammatory response in the brain [74].

In our study, we further demonstrated that four SUS treatments were sufficient to yield a significant reduction in tau pathology, and by combining SUS and the RN2N antibody fragment, we not only achieved increased histological, but also increased behavioral improvements. Importantly, using fluorescently labelled RN2N, we found that SUS not only caused an increased uptake by the brain, but moreover that the antibody fragment was effectively taken up into neurons where the tau damage occurs, and could be visualized not only in the cell body, but also proximal and distal dendrites [71]. This demonstrates that SUS can clear a pathology that, different from the Aβ pathology, is mostly intracellular. Moreover, this study presents SUS as an efficient method to deliver drugs (including different antibody formats) past the BBB into the brain and its cellular constituents.

An alternative approach to achieve therapeutic concentrations of antibodies in the brain is to use viral vectors such as the adeno-associated virus (AAV) vector. Recently, the genes encoding the anti-p-tau monoclonal antibody, PHF1, were delivered directly into brains of P301S mice. In contrast to previous studies using passive immunization with the same antibody, hippocampal antibody levels achieved after AAV delivery were ∼50-fold higher, achieving marked (≥80–90%) reductions in hippocampal tau pathology [75]. AAV-mediated delivery was also demonstrated with a gene encoding an anti-tau scFv in the P301S mice [73]. Although in both studies direct intracerebral injection of the AAV was conducted, a delivery route which is less than ideal for clinical trials, studies aimed at optimizing the vector capsids to efficiently and widely transduce the CNS following intravenous injections have been conducted [76]. Furthermore, ultrasound has been used to successfully enhance the delivery of intravenously delivered AAV across the BBB to achieve widespread gene expression in the brain [77], demonstrating that the technique can additionally be used to facilitate gene therapy approaches for treatment of AD.

CONCLUDING REMARKS: PREDICTIONS

The last years have seen several changes in tau research. Whereas an initial focus has been on serine/threonine-directed phosphorylation (and consequently the kinases and phosphatases that regulate this post-translational modification), tyrosine-directed kinases (such as Fyn and Pyk2) and phosphatases (such as STEP [78]) will be gaining more attention in coming years. Similarly, it can be anticipated that mechanisms in causing tau accumulation that are not driven by phosphorylation and subcellular relocalization will increasingly be explored, and research will be extended to neurodegenerative diseases with protein aggregation other than AD and FTDP-tau. Another shift has occurred with regards to studying the compartment in which tau causes damage. There will be an increasing appreciation that pathological tau impacts all aspects of mitochondrial function. Its role in nuclear functions is also increasingly being explored [79, 80], with more work anticipated to be done in the near future. An integration of how tau impairs the electrophysiological properties of neurons at the synapse and the AIS is still missing, and how this is tied to the release of tau into the extracellular space [81]. Here we do think that more will be learned about the ways in which tau aggregates are being packaged and how the contents is released, is taken up, and interacts with tau in recipient cells. That there is widespread tau seeding

activity in AD at early Braak stages has only been shown recently [82].

It may also be that the roundworm *C. elegans* with its unique capabilities will be more utilized as an animal model complementing rodent work. In *C. elegans*, protein with tau-like repeat-1 (PTL-1) is the sole homolog of tau. We had found in collaborative work that PTL-1 regulates both neuronal and organismal aging [83, 84]. Furthermore, we found that PTL-1 deficient worms are hypersensitive to oxidative stress and are defective in the nuclear accumulation of the transcription factor SKN-1 in response to stress [85]. Interestingly, in mammals, the SKN-1 homolog Nrf2 has been shown to be involved in the autophagic degradation of p-tau [86]. This highlights the possibility to effectively link rodent and worm studies to gain deeper insight into pathogenic mechanisms. More work will finally go into an understanding how Aβ and tau 'talk' to each other. We are still far away from understanding the signaling cascades and integrating the different pathomechanisms that have been claimed to have a role in AD.

As we continue to learn more about the molecular mechanisms underlying the pathogenesis of AD, novel therapeutic targets and strategies for the clearance of specific populations of Aβ and tau will be identified. An example of this is the unpublished work from Karen Duff's group which employs the use of the neuropeptide PCAP to activate the proteasome specifically in dendrites, thereby reducing dendritic tau levels only [http://www.alzforum.org/news/conference-coverage/new-explanation-dendritic-tau-its-made-there]. Another example is the use of gamma frequency (20 to 40 Hz, i.e., at the other end of the spectrum compared to ultrasound) that has been shown to attenuate amyloid load in mouse models [87]. Despite the success of therapeutics delivered via traditional mechanisms in pre-clinical animal models, we envision that the translation of AD therapeutics into future human clinical trials will employ non-invasive, efficient delivery systems to ensure adequate concentrations of therapeutics are reached in the brains of human patients. This will not only reduce the cost of treatment to an amount which will be sustainable by a country's health system, but will also reduce the number of treatments for the patient. We also envision the use of combinatorial drugs, that for example target both tau and Aβ. As far as tau is concerned there will likely be a shift from targeting its posttranslational modifications and the enzymes in charge to simply lowering tau levels. Together, we anticipate a better integration of basic and translational research in order to achieve better health outcomes.

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New Beginnings in Alzheimer's Disease: The Most Prevalent Tauopathy

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Abstract. Alzheimer's disease (AD) is characterized by the presence of two aberrant structures: namely senile plaques, composed of amyloid-β peptide (Aβ), and neurofibrillary tangles, composed of tau protein. In this regard, Aβ and tau protein have been widely studied in research efforts aiming to find a therapy for AD. Aβ and tau pathologies do not always overlap. The precursor of Aβ is expressed in peripheral tissues and in the central nervous system (CNS), whereas tau is mainly a neuronal protein. Since AD is a disease of the CNS, it has been proposed that Aβ may initiate the disease process, with tau being the executor. In this review, we will focus on future studies of tau pathology, although we will comment on new beginnings for AD, as other molecules other than Aβ and tau may be involved in the onset of dementia.

Keywords: Extracellular tau, MAPs, tau functions, tauopathies

INTRODUCTION

One hundred years ago, Alzheimer's disease (AD) was described as a condition involving the presence of senile plaques (Aβ aggregates), neurofibrillary tangles (tau protein polymers), and neuronal death [1]. Thus, the development of Aβ and tau pathologies does not overlap, with Thal stages [2] of the former differing from Braak stages [3] of the latter. In this review, we will focus on tau pathology.

AD is the most prevalent tauopathy; tauopathies are diseases involving a dysfunction of tau protein, through a loss of function or a gain of toxic function. Research involving mouse models revealed that the lack of tau does not cause death or clear neurodegeneration [4, 5]. It is therefore assumed that tauopathies like AD are the consequences of a gain of toxic function [6, 7], which may be related to the accumulation of modified or unmodified tau in neurons, among other features [8].

AD IS CHARACTERIZED BY A HIGHER AMOUNT OF TAU PROTEIN

The brains of AD patients show a greater accumulation of tau protein compared with those of healthy counterparts [9]. This increase in tau may result from an increased transcription of *mapt* gene, an increase in the translation of tau protein, or a deficient tau degradation [10–13]. Among signaling pathways, mTOR may participate in an increased translation and a

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decreased degradation of tau protein. This notion is supported by the observation of increased mTOR signaling in the brains of AD patients [14]. This activation of mTOR may trigger mRNA translation into tau protein through the recognition of a terminal of oligopyrimidine track (5'-TOP) sequence present at the 5'UTR of mapt RNA [10].

On the other hand, mTOR1 activation inhibits autophagy [15], thereby possibly impairing tau protein degradation. In addition, tau expression may depend on tau haplotype (H1 or H2) [16] or on the presence of miRNAs that bind to the 3'UTR mapt mRNA [17, 18]. Regarding protein degradation, AD involves impaired proteasome [11], and modified (aggregated or phosphorylated) tau may inhibit proteasome or autophagy functions. The current working hypothesis is that tau accumulation is caused mainly by deficient protein degradation rather than by an increase in the expression of this protein. A lower tau turnover may facilitate tau phosphorylation by various kinases or its modification by truncation (upon cleavage with several proteases), acetylation, glycation, or other posttranslational modifications that also result in the accumulation of modified tau [8]. Some of these modified forms are toxic when present in neurons. Thus, the long life (due to a lower turnover) of intracellular tau may have negative consequences. In addition, distinct ratios of tau isoforms containing three (tau 3R) or four (tau 4R) tubulin-binding repeats, arising by a different splicing of nuclear *mapt* RNA, could result in a toxic effect promoting tauopathies such as Huntington's disease [19]. Splicing mechanisms can give rise to a number of different tau isoforms [20], and their involvement in neuronal toxicity deserves further attention.

HOW DOES THE BRAIN DEAL WITH AN INCREASE IN INTRACELLULAR TAU IN AD?

Since the proportion of tubulin in brain is much higher than that of tau protein (or other microtubule-associated proteins, MAPs), a slight increase in brain tau can result in an additional interaction of the protein with the available open sites present in neuronal microtubules. This interaction will lead to a greater tau/tubulin ratio in polymerized microtubules when the increase in tau is through an excess of the functional unmodified form. In this case, the increased in tau also leads to competition with other molecules or organelles (like mitochondria) for the same microtubule binding sites [21]. Such competition may affect the transport (mediated by microtubules) of these organelles, in a similar way to the effect found in other MAPs [22].

A further increase in neuronal tau can lead to a change in the subcellular localization of this protein. Tau is preferentially distributed in the axonal compartment [23], but an increase in this protein favors its localization to somatic dendritic compartments [24]. In addition, an increase in the level of intracellular tau may result in its secretion to the extracellular space [25], where it can be toxic for neighboring neurons and can propagate throughout the brain [26, 27]. In this case, tau might be secreted in an unmodified or modified (truncated or aggregated) form [28].

One strategy through which to tackle the increase in intracellular tau is to reduce its expression or to increase its degradation by acting on mTOR pathway. Alternatively, tau expression could be decreased by increasing the expression of miRNAs, like miRNA129, thereby reducing its translation [18].

HUMAN TAU AND TAU OF OTHER ORIGINS: DO THEY PLAY A DIFFERENT ROLE IN TAUOPATHIES?

A review entitled "The exceptional vulnerability of humans to Alzheimer's disease" has recently been published [29]. This review reports that an increased vulnerability of human tau, compared with tau proteins from other sources, cannot be discarded. In this regard, it is therefore pertinent to study not only the increased expression of tau but also its structural nature. Several studies have been carried out to compare the structural differences or changes in posttranslational modifications of tau protein of distinct origins [30, 31]. Some studies have also addressed changes in posttranslational modifications, but further analysis is required to gain a broader understanding of this point [32].

EXTRACELLULAR TAU

As previously indicated, an increase in the level of intracellular tau results in its secretion [25] or, in a few cases, neuron death [26]. In both scenarios, it also leads to the presence of extracellular tau, a toxic molecule [26]. Various mechanisms of tau exocytosis (secretion) have been proposed [32–35]. In some cases, soluble unmodified tau is secreted while in others modified (truncated, phosphorylated,

aggregated, etc.) tau is released from the cell. Such release is through a naked form or through exosomes [25]; however, it has also been put forward that this release occurs through tunneling nanotubes [34]. Extracellular tau also interacts with surrounding neurons and can be internalized via various endocytotic pathways. Depending on whether tau is in an unmodified or modified form, it binds to cellular receptors (muscarinic receptors M1/M3) [27] or to components of the extracellular matrix, like heparan sulphate [33], respectively. Also, research efforts should address whether other mechanisms of endocytosis are involved [36].

In addition, extracellular tau interacts with glial cells. In this regard, mainly the interaction of tau with microglia has been analyzed [37], and preliminary data suggest that this interaction occurs from different receptors to those previously described for neurons, despite the presence of muscarinic M3 receptors in a small population of microglia [38]. Also, some components of the extracellular matrix, like heparan sulphate, are present in microglia. However, results from preliminary studies support the notion that a novel tau receptor is located in resting microglia.

Thus, an increase in intracellular or extracellular tau may have negative consequences. In the case of extracellular tau, it can be cleared through the action of microglia; however, these cells lose some of their functional characteristics in tauopathies like AD [39]. Although blocking the cellular receptors needed for tau binding has been proposed [27], current research efforts are focused on the development of tau vaccines [40, 41]. Future studies are expected to determine the potential of these vaccines to prevent the toxicity and propagation of extracellular tau.

CONSEQUENCES OF TAU ELIMINATION IN NEURONAL CELLS

We have previously proposed that therapeutic strategies for tauopathies like AD should involve reducing the level of intracellular tau or clearing extracellular tau. In this regard and given that mouse models have revealed that the absence of the protein does not affect viability or stimulate neurodegenerative disorders [4, 5], one therapeutic approach could be to remove the whole tau protein. However, the absence of tau may result in the loss of some functional characteristics of tau-deficient mice.

It has been proposed that intracellular tau exerts several functions. For example, tau protein, which is a MAP, favors the assembly of microtubules *in vitro* [8]. Its presence results in decreased microtubule dynamics and an increase in microtubule stability [42]. Also, tau regulates the number of protofilaments in microtubules [43]. In contrast to other proteins, like EB proteins, which bind at the GTP-tubulin-rich microtubule tips, tau shows greater binding affinity to GDP like-tubulin conformations [44]. On the other hand, the cross-talk of tau with EB proteins has been shown to regulate axon extension in developing neurons [45]. Also, interaction between EB1 and tau protein is postulated to regulate axonal tau sorting [24]. However, some of these tau functions are complemented in tau knockout mice by the presence of other proteins and neuron differentiation is delayed but not impaired in tau-deficient mice [5].

More specifically, the loss of tau results in an increase in wakefulness duration and decreased NREM sleep [46]. Also, tau knockout mice show shaking and other features of Parkinsonism [47, 48], In addition, these animals exhibit brain insulin resistance [49] and alterations of cardiovascular functions [50].

A main consequence of tau loss has been found at the apical dendrites of newborn granule cells present in the dentate gyrus. In tau knockout mice, dendritic spines do not grow when the mouse is exposed to an enrichment environment (which usually occurs in wild-type mice). Also, the loss of dendritic spines in these apical dendrites in wild-type mice under stress is not observed in the tau knockout model [51]. These results indicate a novel function of tau protein related to synaptic plasticity, thereby suggesting that this molecule is a synaptic plasticity modulator for positive or negative external stimuli [51].

Furthermore, it is known that the presence of tau in dendritic spines regulates the toxic effect of Aβ in neurons [52]. In this regard, Aβ peptide, Glu N2B (a subunit of NMDA receptor), tyrosine kinase fyn, and PSD-95 (postsynaptic protein) are involved in this process [52]. Despite the action of Aβ, it has also been proposed that tau-fyn-GluN2B regulates the activity of CREB, a protein related to memory and learning [53].

Since AD is considered a synaptopathy, in-depth analysis of the role (positive and negative) of tau in synaptic connections is required. Independently of tau, the use of compounds to prevent synaptic deficits is not straightforward, since many pharmaceutical agents should not cross the blood-brain barrier (BBB). Nevertheless, some BBB-permeable

compounds, like a modified peptide of the ciliary neurotrophic factor, can rescue synaptic deficits [54].

FUTURE DIRECTIONS AND NEW BEGINNINGS

Regarding tau pathology, research appears to be focused on ways to decrease the level of intracellular tau—mainly the toxic modified tau forms (phosphorylation, truncation, aggregation, etc.)—in neurons. In the case of extracellular tau clearance, the development of vaccines emerges as a major objective [40]. However, such vaccines should be administered at the most appropriate stage of AD development. In this regard, this disease is characterized by three developmental stages: an asymptomatic stage, related to amyloid pathology; a transition step from non-demented to mild cognitive impairment, related to tau pathology; and a third stage involving the development of dementia and related to neuron death and glia activation (inflammation). Once tau pathology is evident, the use of compounds against amyloid pathology is probably no longer suitable. Also, after neuron death, the use of compounds against tau pathology could be useless. It is therefore important to treat each pathology in a timely manner. To achieve this, it is necessary to have access to early biomarkers, thereby allowing treatment at the onset of the disease.

Moreover, the therapeutic focus in AD has fallen mainly on two targets, namely Aβ and tau. However, other targets that remain to be identified may facilitate the onset of the disease. In this regard, potential new targets deserve attention.

These possible novel factors include brain somatic mutations that may be related to processes associated with Aβ or tau pathologies. In this regard, some reports have described single nucleotide variations in brain tissue of AD patients [55, 56]. Also, inserts, deletions, and transposons [57–59] related to the appearance of AD deserve attention. Also, further analysis should be devoted to epigenetic changes [60], in the search for alternative therapies [61].

Finally, this short summary makes no reference to damage in neuronal circuits or complementation between circuits (which could delay the appearance of the disease) [62], the possible deficits related to the disease that correlate with impaired adult neurogenesis [63], or attempts to delay the onset of the disease by slowing down the aging process, since the main risk for AD is aging [64].

DISCLOSURE STATEMENT

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Tau Conformation as a Target for Disease-Modifying Therapy: The Role of Truncation

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Abstract. Tau protein plays a major role in the pathogenesis of Alzheimer's disease. Despite many decades of intensive research, the cause of the conformational switch that leads to the remodeling of the highly flexible conformational ensemble of intrinsically disordered protein tau into insoluble filaments is still elusive. We show here that truncation of tau may play a causative role in this conformational change, as evidenced by results obtained from *in vitro* experiments and from transgenic animal models. This conformational change is a common denominator of pathological tau protein assemblies, and a salient drug target. The long-running research of truncated tau has led to the generation of the first active tau vaccine that has entered clinical trials.

Keywords: Aggregation, Alzheimer's disease, conformational ensemble, immunotherapy, tau protein, truncation

INTRODUCTION

Ever since cognitive loss and dementia at an advanced age were understood to be due to a pathophysiological process, and not a natural part of aging, therapeutic efforts for neurodegenerative disorders have been both intense and diverse (see [1] for a recent review).

Tau

The key role of tau protein in Alzheimer's disease (AD) was obvious and plain to see; no AD patient has developed dementia without extensive neurofibrillary pathology, and vice versa, patients at a Braak stage of 5 or 6 are rarely, if ever, cognitively intact [2] (for the rare occurrences of high Braak stages being assigned to cognitively normal individuals, one has to ask the question whether the score was assigned based on an isolated tangle in a brain region that usually develops pathology at later stages). Neurofibrillary pathology was found to correlate with the severity of dementia, and its distribution with the phenotype of cognitive impairment and affected domains of cognition [3–5]. The case for tau as a driver of neurodegeneration is even clearer in the various tauopathies, where pure tau pathology, often tied to a MAPT mutation, leads to neurodegeneration [6, 7]. Recent research has also revealed the most likely mode of propagation of neurofibrillary lesions, a prion-like spreading via "tauons", intercellularly transmissible tau moieties that serve as templates for tau aggregation [8], resulting ultimately in the deposition of tau in filaments with high beta sheet content [9]. While these tauons constitute natural drug targets, how they come into being is anybody's guess though, and the scientific

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community is far from unified in regard to their key features and common denominators.

Then, what is there to a tauon, what key features? What can be used to tell it apart? First of all, it is important to highlight that, opposed to the 6 isoforms seen in health, the diseased tau proteome is *extremely diverse*, even at the level of protein sequence: as a result of truncation, fragments of various length arise [10]. Fragments containing the N-terminus but without the microtubule-binding repeats (MTBR) seem to preferentially find their way into the cerebrospinal fluid (CSF) [11], whereas all aggregating tau species participating in the formation of neurofibrillary pathology have at least a portion of the MTBR intact [9, 12]. Aggregation is an essential part of prion-like template-mediated conformational change, thus all tauons contain the MTBR or a part thereof. Truncation was shown to greatly promote the aggregation of tau [13], but the neo-epitopes created by truncation may not be the best immunotherapy targets, as even pathological tau molecules can become even further truncated, losing these epitopes in the process [14] (Fig. 1).

A further layer of diversity is provided by various other post-translational modifications of tau—ubiquitination, nitration, glycation, O-GlcNAcylation, or phosphorylation. Using phosphorylation as an example, the variability of tau becomes apparent once we consider that of the protein's 441 amino acids (in the 2N4R isoform), roughly 80 are serine, tyrosine, or threonine that can be phosphorylated. The phosphorylation is subject to a vigorous flux, with kinases attaching phosphates, and phosphatases (e.g., PP2A) wiping the phosphates away again. It is clear that tau is excessively phosphorylated in AD [15], and it is likely that disturbance of the phosphorylation-dephosphorylation cycle can cause tauopathy, e.g., the Parkinsonism-Dementia complex of Guam [16]. The most relevant question, though, is whether any given phospho-epitope is present in all tauons, or at least a significant portion thereof.

To summarize: 1) all tauons will inevitably contain the MTBR, 2) they may or may not possess one or both intact termini, though truncation is widely prevalent, 3) they are likely to possess excessive phosphorylation and other post-translational additions, but the pattern is likely not uniform, and 4) they consist likely of multiple aggregated tau molecules (some studies report the smallest stable unit to be an 3-mer [17], though tau monomers possess pro-aggregant traits, and were shown to be able to attain and maintain pathological conformations following truncation).

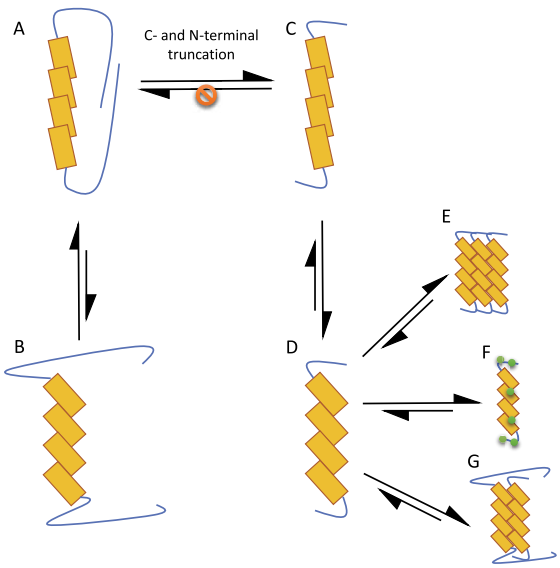

Fig. 1. Impact of truncation on the conformation of tau. Equilibrium between the healthy form of full-length tau (A) and a misdisordered form [56] of healthy tau (B) is shifted toward the healthy form, whereas for truncated tau (C, D) the opposite holds true: the misdisordered form (D) is the energetically preferred state. The conformational ensemble of truncated tau has a far greater accessibility of its MTBR and this increases its propensity for homooligomerization (E), hyperphosphorylation (F), and aggregation even with healthy tau (G).

Tau-targeted therapies have taken both the small-molecule and immunotherapy approach.

- Small-molecule approaches were focused on inhibiting tau aggregation, e.g., methylthioninium [18], taxanes and other microtubule stabilizers to counteract cytoskeletal destabilization caused by pathological tau [19, 20], kinase inhibitors to reduce tau phosphorylation [21], or neurotrophic peptides with anti-phosphorylation properties (davunetide) [22]. None of these approaches were successful as of today.
- The first tau-targeted immunotherapy, AADvac1, has entered clinical development in 2013 [23]; the compound stimulates the production of antibodies against a phosphorylation-independent conformational epitope found in the MTBR of tau. These antibodies are expected to prevent tau aggregation, intercept tauons, and opsonize them, so that they are taken up by microglia and removed (see Fig. 3).
- Multiple tau-targeted immunotherapies have since then entered clinical development:

- ACI-35, an active immunotherapy raising antibodies against the pS396/pS404 epitope, hypothesized to target extracellular spreading tau [24];
- BIIB092, a humanized IgG4 monoclonal antibody targeted against extracellular N-terminal tau fragments [25];
- C2N 8E12, a humanized monoclonal antibody targeted against extracellular tau [26];
- RG7345, a humanized monoclonal antibody targeting the tau phospho-epitope pS422. Unlike the other immunotherapies discussed here, the antibody primarily aims to target intracellular tau [27]. The development of RG7345 was discontinued for undisclosed reasons.

The indications for which these compounds are being developed follow clear trends:

- AD as the most common tauopathy.
- PSP as a pure tauopathy with high phenotype-pathology correlation, a good diagnostic accuracy, and swifter progression than AD (allowing shorter time frames for clinical efficacy readouts) [28].
- Few tau-targeted compounds, with the notable exception of LMTM [18], were tested in the behavioral variant frontotemporal dementia; the indication has the major drawback that tau pathology underlies only ~50% of bvFTD cases, and the other 50% display mostly TDP43 pathology, but neither the phenotype nor imaging or CSF biomarkers are sufficiently informative about which pathology is present in a given case [29].
- nfvPPA, a phenotype of primary progressive aphasia mostly associated with tau pathology is a recent target of tau-targeted therapy (NCT03174886); the indication's main appeal is the fact that the progression of language-dominated symptoms (and thus the impact of therapies) is assessable even while patients are non-demented; also, the phenotype is initially free of motor symptoms that would limit survival, again facilitating trial conduct [29, 30].
- MAPT mutation carriers constitute a natural population for the testing of tau-targeted therapies, but their numbers are severely limited; also, tau mutations are very variable in their age of onset and symptom presentation [31].
- Corticobasal syndrome as a 4-repeat tauopathy is also a potentially suitable indication for the development of tau-targeted therapies; the link between the CBS clinical phenotype and the corticobasal syndrome pathology is weaker than in the case of PSP, though [29]. In some studies, patients with either indication are enrolled to increase recruitment (e.g., NCT02133846).

Amyloid-β

Due to the fact that a small fraction of AD cases are caused by monogenic mutations in amyloid-β protein precursor (AβPP) or the presenilin 1 and 2 enzymes involved in its processing, the amyloid hypothesis of AD has received massive attention. Similarly, the fact the *APP* gene is located on chromosome 21, which is present in triplicate in Down syndrome patients who suffer from progressive neurodegeneration, has lent further support to the hypothesis [32]. Therapeutic approaches targeting basically all aspects of the proposed amyloid cascade have been tested, e.g.:

- Immunotherapy aimed at removing the amyloid-β (Aβ) peptide:
  - active vaccines, e.g., AN1792 [33] and CAD106 [34];
  - passive vaccines, e.g., bapineuzumab [35], solanezumab [36], gantenerumab [37], aducanumab [38].
- Amyloid aggregation inhibitors [1].
- Molecules affecting the enzymes involved in AβPP processing (secretases), aiming to reduce production of Aβ [1]

Despite intense efforts, no anti-amyloid treatment was proven to be efficacious yet, even in cases where extensive clearance of amyloid deposits was achieved [35]. Furthermore, intervening in amyloid processing was found to be a non-trivial matter, as the various enzymes involved possess other vital functions, and interfering with them results in very poor safety profiles [39, 40].

The current consensus amongst proponents of the amyloid hypothesis appears to be that Aβ just initiates the AD pathophysiological process, and if an anti-amyloid strategy is to be effective, intervention is necessary prior to widespread generalization of neuropathology. As a result, anti-Aβ therapies are shifting their attention to ever-earlier stages of AD (e.g., DIAN, A4, API). Important questions, such as why massive amyloidosis without tau pathology is asymptomatic, still remain unanswered [4].

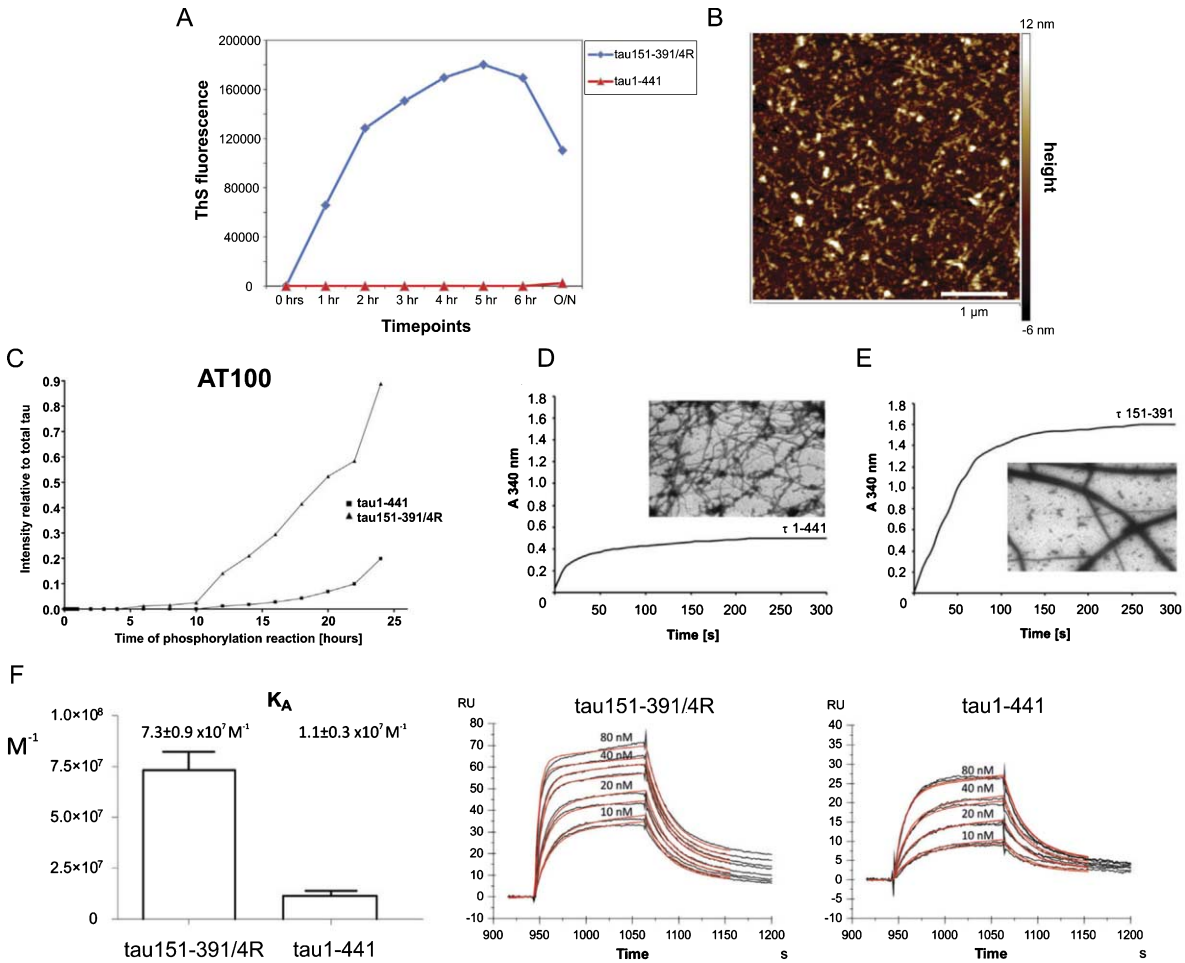

Fig. 2. Experimental characterization of truncated tau151-391/4R. A) Heparin induced oligomerization reaction of truncated and full length tau monitored by ThS fluorescence, O/N-overnight. B) AFM image of the product of 48 hours long heparin induced tau oligomerization reaction of tau151-391/4R. C) Phosphorylation reaction of AT100 epitope [74]. D, E) Microtubule assembly assay monitored by increase in OD at 340 nm and EM images of microtubules induced by full length and truncated tau (adapted from [70]). F) K_A values and SPR sensorgrams for the interaction of DC8E8 antibody with full length and truncated tau (adapted from [54]).

Other approaches

Covering every single facet of AD therapy development is beyond the scope of this article. Suffice to say, as diverse as the hypotheses about pathophysiology of AD are, so diverse are the treatment approaches. Neuroinflammation is a salient feature of AD [41] and numerous anti-inflammatory approaches have been tried in the clinic (anti-inflammatory drugs, statins, etc.). As aging is the primary risk factor for AD, numerous studies were conducted on nutrition supplements, hormones, and trophic factors in an attempt to slow or reverse brain aging [1]. Finally, non-pharmacological approaches, comprising optimization of dietary, blood pressure medication, and diabetes therapy, and intense physical and cognitive exercise were found to reduce dementia incidence in an at-risk elderly population [42], indicating that they will be a valuable companion therapy to disease-modifying agents once these are developed.

EVIDENCE FOR PATHOPHYSIOLOGICAL TAU TRUNCATION IN VIVO

Truncated tau proteins were initially identified as constituents of the pronase-resistant paired helical filament (PHF) core [43, 44]. The first evidence of tau truncation in the AD brain was obtained via the monoclonal antibody MN423, which recognizes tau

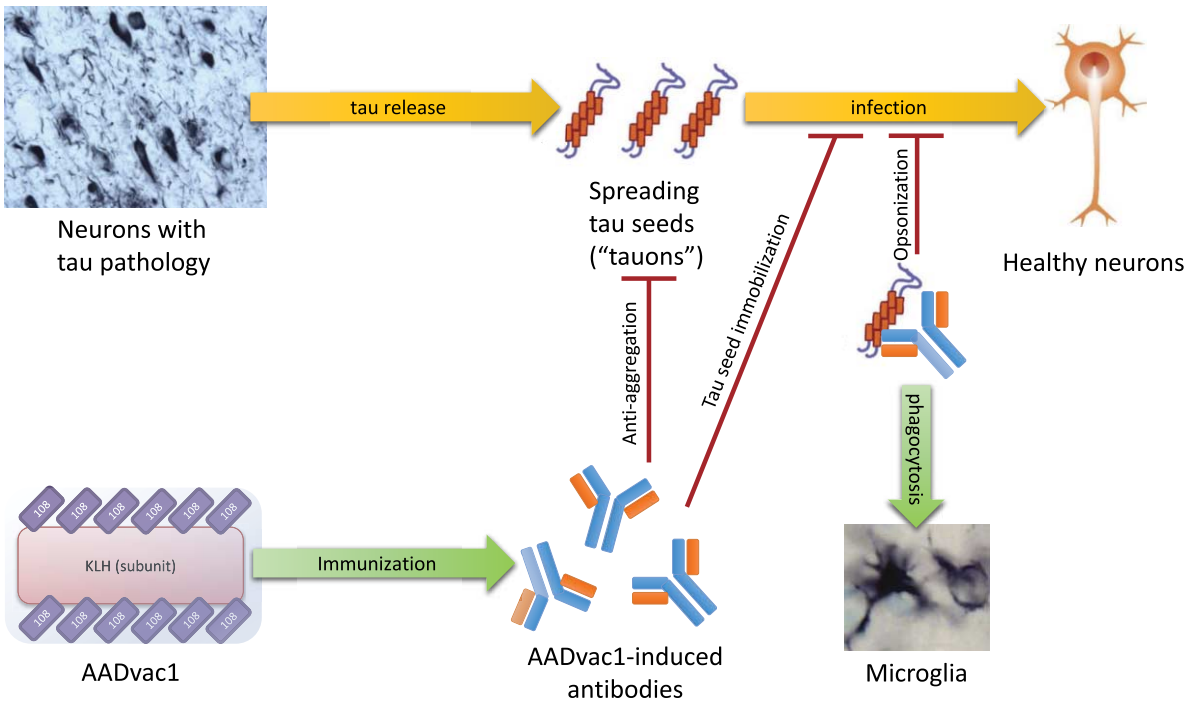

Fig. 3. Proposed mechanism of action of AADvac1. AADvac1 leads to the production of antibodies that prevent tau aggregation, immobilize tauons, and flag them for removal by the immune system.

proteins truncated at Glu391 [12]. Later, it was found that tau protein is cleaved by caspases at several sites, especially at Asp421 by caspase-3 [45, 46]. The disease specificity of certain truncation/conformation patterns becomes apparent when analyzing the brain via antibodies such as the truncation-dependent conformational antibody DC11, which recognizes solely conformationally modified tau proteins from AD brains and does not recognize tau proteins from healthy brains. Its AD-specific conformational epitope can be reconstituted *in vitro* by truncation of recombinant tau, indicating that disease-specific tau conformations arise naturally when tau is truncated [47, 48].

Zilka et al. have performed tandem-affinity purification of the sarcosyl-insoluble protein fraction from the human AD brain (Braak V) using antibodies specific for extreme N-and C-termini of tau and have shown the presence of both N- and C-terminally truncated forms of tau protein, including the previously identified tau fragment that constitutes the core of PHF. The presence of this fragment (dGAE) was verified with MS analysis and proves its presence in pronase-untreated PHFs, resolving the long-running debate whether tau truncation is an artifact of pronase treatment, or arises naturally in disease [10].

Derisbourg et al. have identified several new N-terminal truncation sites using LC-MS/MS analysis of brain extracts from controls and AD patients after immunoprecipitation with the Tau5 antibody (epitope 218–225). They have chosen truncation sites Met11 and Gln124 for further biochemical analysis. The tau fragment starting at Gln124 showed a stronger ability to bind microtubules and protect them from depolymerization compared to full length tau 1N4R. This effect can lead to impaired synaptic plasticity, and to wasteful and inefficient microtubule assembly. The phosphorylation status was evaluated after transfection of corresponding expression vectors into N1E-115 neuroblastoma cell line and subsequent western blot analysis after 48 hours. The construct starting at Met11 displayed an increase in phosphorylation at the Thr231 epitope, and no difference in phosphorylation at Ser396 compared to full length tau 1N4R. Interestingly, the construct starting at Gln124 displayed a decrease in phosphorylation at Thr231 and Ser262/356 compared to full length tau 1N4R, indicating that truncated tau molecules possess different propensities toward (hyper)phosphorylation [49].

EFFECT OF TRUNCATION ON THE STRUCTURAL PROPERTIES OF DISORDERED TAU MOLECULE: PATHOLOGIC TOXIC GAIN OF FUNCTION

Tau is a typical intrinsically disordered protein (IDP) [50, 51]. The structure of an IDP cannot be described by a single conformation, but rather by a set of different conformational states, commonly designated a 'conformational ensemble' (CE). Each member of the IDP CE occupies one of the local energy minima on the energetic landscape, with low barriers between them [52]. Similarly, an individual conformational state can be seen as a sub-set of the CE, whose members are freely interconverting conformers. Taken together, the CE of an IDP is defined by its individual members and by the distribution of IDP molecules between them [50]. It has to be underlined that the biological activity of IDPs is completely determined by the composition of their CE, which is encoded by IDP sequence, posttranslational modifications, environment, binding partners, etc.

It was observed that IDPs can support several non-standard modes of allosteric regulation, consisting generally of a conformational remodeling of their CE after a signal-inducing event, such as posttranslational modification (e.g., phosphorylation), binding of a small ligand or other molecule, etc. [53]. This conformational remodeling consists of a repopulation of individual conformational states and changing the distribution of IDP molecules between them. For example, augmenting a state that features an exposed signal-transduction domain may kinetically boost IDP binding to receptors that have an affinity for said domain, and change the protein's interactome considerably.

Intriguingly, truncation of tau emerges as an up to now overlooked inducer of tau CE remodeling. We have observed that truncation of both the 3R and 4R tau isoform results in an order of magnitude faster binding to conformational monoclonal antibody DC8E8 [54]. Faster binding reflects a greater accessibility of the DC8E8 epitopes that are located in the microtubule-binding repeat domain and indicates a change in the population of the CE of truncated tau in comparison to the CE of the full-length isoforms. As the DC8E8 epitope lies in the vicinity of the aggregation-prone tau domain, its exposure means that truncated tau has a lower entropic barrier to self-association, i.e., greater accessibility of the β-sheet forming domains, which inevitably fosters tau-tau interaction. These results are in agreement with the over-representation of truncated tau form in neurofibrillary pathology, and with the higher aggregation tendency of truncated tau (see below). Furthermore, we have observed in both primary rat neurons and human neuroblastoma cells that truncated tau lacking 150 N-terminal residues has constitutive access to the nucleus (unlike its full-length counterpart whose access is situational), where it engages in interactions with subnuclear structures [55]. Translocation into the nucleus is likely driven by the remodeled CE of truncated tau. We term the remodeled tau ensemble that's in the process of transitioning from a soluble disordered protein to its insoluble, misordered aggregated form the "misdisordered" state of tau [56].

Truncated tau has been suggested to trigger neurofibrillary degeneration [12] and to drive the pathological conversion of wild-type tau at neuritic plaques [57]. *In vitro* tau aggregation studies with inducers of tau polymerization have shown that both C-terminal truncations of tau at Glu391 and at Asp421 lead to proteins more prone to aggregation than full length tau [58–60]. Also, truncated tau151-391/4R aggregates more rapidly than full length tau upon addition of a polyanionic inducer (B. Kovacech, unpublished results, Fig. 2A). Some truncated tau variants aggregate readily and form PHF-like fibrils also without the addition of an inducer. This was shown for the PHF core tau fragment dGAE (tau297-391/4R) [61]. At low concentrations of dGAE, an inhibitory effect of disulfide bridge crosslinked dimers was observed, which was overcome at concentrations higher than 100 μM [61]. The aggregation without an inducer was also observed for the mutated tau fragment K18Δ280 (tau243-372/4RΔ280) and tau fragment K12 (243-394/3R) [62].

Higher propensity of truncated tau for aggregation may lead to increased oligomer formation and cell to cell spreading, as tau oligomers were detected in mouse models expressing truncated tau protein. Experimental evidence clearly shows that tau truncation is a key step in the induction of tau pathology [63, 64]. According to the prion-like model of tau propagation, tau aggregates formed in a cell are released into the extracellular space, from which they are taken up into other cells, probably via the interaction with cell surface heparan sulfate proteoglycans that stimulate macropinocytosis [65]. The propagation may also occur trans-synaptically and/or via exosomes [66]. In the extracellular space, tau can be a target of matrix-metalloproteinases. *In vitro* it was shown that cleavage of tau by matrix-metalloproteinase 9

enhances formation of tau oligomers and tau fragments 204–330 or 262–391 containing parts of the tau microtubule-binding repeat region [67]. Moreover, a highly-complex polyanionic extracellular matrix may catalyze nucleation of tau oligomers [68]. The presence of tau oligomers in the interstitial space was shown by *in vivo* micro-dialysis from the rTg4510 mouse brain with a large-pore probe [69]. These findings lend themselves to the conclusion that the interstitial space can promote both tau truncation and oligomerization.

DC11 positive N- and C-terminally truncated tau proteins (except for tau99-441) exert 3-4 times higher microtubule assembly activity than full length tau and produce malformed, abnormally thick microtubule bundles [70]. Microtubule bundles were observed also in the presynaptic terminals of transgenic animals expressing tau151-391/4R; in this model, truncated tau was shown to deregulate synaptic markers in presynaptic compartments [71].

Expression of truncated tau151-391/4R in SH-SY5Y neuroblastoma cells induces caspase-3 independent apoptosis-like programmed cell death. The expression of truncated tau was significantly more toxic for the cells than the expression of full length tau [72]. It was also shown that expression of truncated tau in this cellular model suppresses the activity of the proteasome, thus inhibiting its own degradation [73].

The monitoring of simultaneous *in vitro* phosphorylation reactions of full length tau and truncated tau151-391/4R with a brain extract has shown that truncated tau is phosphorylated more rapidly and to higher extent than full length tau on several AD relevant phospho-epitopes (AT270, pS199, pT212, pS214, pS262, pS356, AT8, AT100). Particularly the AT100 epitope appears on truncated tau after 8 hours of reaction and the increase of its intensity on western blot is exponential, whereas on full length tau it is formed after 14 hours, with slow increase (Fig. 2C). This shows that truncation of tau leads to the change of conformation that is more accessible for kinases [74].

PHENOTYPE OF TRANSGENIC MODELS EXPRESSING TRUNCATED TAU PROTEIN

Modelling neurofibrillary pathology in transgenic models with full-length tau is hardly achievable without introducing a point mutation [75]. On the contrary, models with truncated tau protein as transgene easily reproduce pathological aspects of human tauopathies (for review, see [64]). The first rat model that established tau truncation as a factor sufficient to drive neurofibrillary degeneration in the absence of a tau mutation, transgenic line SHR318, was created by expressing tau 151-391/4R under the control of the mThy1 promoter [70]. More than 15 transgenic models created since then confirmed that animal models based on truncated tau reproduce pathological aspects of human tauopathies much more easily than those using full-length tau.

Truncated tau in transgenic models induces a type of pathology highly similar to AD, starting with the formation of progressively phosphorylated tau oligomers at a pre-tangle stage, and ending with insoluble tangles; these tangles are thioflavin-S reactive, Congo-red birefringent, and argyrophilic, thus displaying all signs of tangle maturity. Perhaps most importantly, truncated tau transgenes are able to sequester full-length endogenous rat tau into high-molecular weight aggregates, unlike other commonly used models where the pathology is composed solely of the transgenic tau [63, 70, 76–81].

Neurotoxicity of truncated tau is reflected in various neurobehavioral phenotypes, like motor impairment [77, 81–87] and deficiency in short-term memory and spatial learning tasks [63, 77, 82, 85–87].

Truncated tau transgenic models have been used for investigation of the changes in CSF due to neurofibrillary degeneration, leading to the proposal of various tauopathy CSF markers, namely metabolites [88], peptides [89], amino acids, [90, 91], and neurotransmitters [92]. Response to stress and the interplay between stress, neuroinflammation, and neurodegeneration has been extensively studied on truncated tau models as well [93–95].

THERAPEUTIC APPROACHES TARGETING THE MISFOLDING OF TAU

A number of active vaccines targeting tau protein has been proposed. The first to be used in humans is the active vaccination with the N-terminally cysteinylated tau peptide ^{294}KDNIKHVPGGGS^{305} coupled to KLH, designated 'AADvac1'. It is designed to induce the production of antibodies against a newly identified domain that regulates tau oligomerization [96]. Safety, tolerability, and efficacy of AADvac1 are being evaluated in ongoing clinical trials [23]; NCT02579252; NCT03174886.

The component of AADvac1 that is designed to induce an antibody response against pathological

tau protein, i.e., the peptide $tau_{294-305}$ is derived from the epitope of the monoclonal antibody DC8E8 [54]. DC8E8 differs markedly from other tau-targeted immunotherapies in development in several aspects, including its ability to 1) inhibit tau-tau interaction, 2) bind tau at four different epitopes in the microtubule-binding repeat domain (MTBR), and 3) selectively recognize conformationally aberrant (misfolded) species of tau that are likely the driving force behind template-mediated tau aggregation and disease progression in AD and non-AD tauopathies [54, 97] (Fig. 3). The four homologous sequences in the MTBR of tau targeted by DC8E8 and by AADvac1-elicited antibodies (further referred to as "DC8E8 tetratope") with the common amino acid pattern HxPGGG [54] are strategically placed throughout the domain of tau that's essential for its assembly into filaments. The first and the second epitopes of the DC8E8 tetratope strategically precede the polymerization-prone, β-structure forming motifs ^{275}VQIINK^{280} and ^{306}VQIVYK^{311}, respectively, which are considered the sites at which tau oligomerization is initiated [12, 98, 99].

In tau transgenic mice and rats, administration of DC8E8 and its active vaccine counterpart AADvac1 respectively led to a reduction in tau pathology, with decreased number of neurofibrillary tangles and depletion of the sarcosyl-resistant tau aggregates; a salient point is that while the targeted epitope is phosphorylation-independent, AADvac1 and DC8E8 treatment led to a pronounced reduction in hyperphosphorylated tau as well, highlighting that conformationally altered tau protein is most likely to become hyperphosphorylated [54, 96]. Considering its efficacy and binding properties, DC8E8 is a suitable candidate for clinical development, defining a new class of disease-modifying passive immunotherapeutics of AD.

Initial clinical results of AADvac1 are encouraging as well. The safety profile was very benign, indicating that tau pathology can be safely targeted in humans. No meningoencephalitis was observed; neither did AADvac1 treatment cause microbleeds or edema (ARIA-E, ARIA-H) that prove dose-limiting for many anti-amyloid therapies.

The vaccine was able to elicit an IgG antibody response against the tau peptide component in 29 of 30 elderly patients; in at least 25 of those patients, the antibody response was shown to target also truncated pathological tau protein 151-391/4R [23]. Perhaps most importantly, the induced immune response recognized tau protein extracts from AD brains in a titer-dependent manner, and the response from individual patients could detect pathological tau in all tested brain extracts, again highlighting the fact that the conformational epitope targeted by AADvac1 is a *conditio sine qua non* of tau aggregation.

Phase II results from tau-targeted immunotherapies are expected to become available in the near future (2019 and onwards).

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The Conundrum of GSK3 Inhibitors: Is it the Dawn of a New Beginning?

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Abstract. Spanning over three decades of extensive drug discovery research, the efforts to develop a potent and selective GSK3 inhibitor as a therapeutic for the treatment of type 2 diabetes, Alzheimer's disease (AD), bipolar disorders and cancer have been futile. Since its initial discovery in 1980 and subsequent decades of research, one cannot underscore the importance of the target and the promise of a game changing disease modifier. Several pharmaceutical companies, biotech companies, and academic institutions raged in a quest to unravel the biology and discover potent and selective GSK3 inhibitors, some of which went through clinical trials. However, the conundrum of what happened to the fate of the AstraZeneca's GSK3 inhibitors and the undertaking to find a therapeutic that could control glycogen metabolism and aberrant tau hyperphosphorylation in the brain, and rescue synaptic dysfunction has largely been untold. AstraZeneca was in the forefront of GSK3 drug discovery research with six GSK3 drug candidates, one of which progressed up to Phase II clinical trials in the quest to untangle the tau hypothesis for AD. Analysis of key toxicity issues, serendipitous findings and efficacy, and biomarker considerations in relation to safety margins have limited the potential of small molecule therapeutics as a way forward. To guide future innovation of this important target, we reveal the roller coaster journey comprising of two decades of preclinical and clinical GSK3 drug discovery at AstraZeneca; the understanding of which could lead to improved GSK3 therapies for disease. These learnings in combination with advances in achieving kinase selectivity, different modes of action as well as the recent discovery of novel conjugated peptide technology targeting specific tissues have potentially provided a venue for scientific innovation and a new beginning for GSK3 drug discovery.

Keywords: Alzheimer's disease, drug targeting, tau

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ALZHEIMER'S DISEASE AND GSK3

Alzheimer's disease (AD) is one of the most common forms of dementia and appears to increase exponentially with age [1]. The etiology of AD has many facets and only a very minor component is

attributed to familial AD of genetic origin [2]. AD is characterized by a progressive loss of episodic memory and cognitive and behavioral dysfunction. One of the most affected brain structures is the entorhinal cortex hippocampus circuitry which plays a key role in memory acquisition and consolidation [3]. Impairments of these brain structures in AD are believed to underlie the impairments in memory that characterize this chronic neurodegenerative disease.

Glycogen synthase kinase 3 (GSK3) has been regarded as a critical molecular link between the two major histopathological hallmarks of the disease, extracellular plaques which are composed of the protein amyloid-β (Aβ) and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein [4, 5]. GSK3 is a highly conserved protein-serine/threonine kinase that was first isolated from skeletal muscle as one of several enzymes that phosphorylated the enzyme glycogen synthase [6]. In mammals, GSK3 is encoded by two highly related genes encoding GSK3α and GSK3β, respectively; however in the brain, GSK3β isoform acts as a key switch that controls numerous signaling pathways [7]. The dysregulation of this kinase has been linked to the development of AD and related dementias, cancer, type 2 diabetes, schizophrenia, depression, and bipolar disorder. Given its relevance in pathophysiological processes, GSK3β is widely considered a therapeutic target of interest [8, 9].

GSK3 activity is regulated by phosphorylation of the Tyr^{279}/Tyr^{216} residue which is important for enzymatic activity [10, 11]. In contrast, inactivation of GSK3 can be achieved through phosphorylation of Ser^{21}/Ser^{9} residues within the N-terminal domain on GSK3, respectively [12]. GSK3 is also regulated upon interaction of the Wnt ligand, its receptor Frizzled and co-receptor LRP5/6. This interaction releases GSK3 from a multi-protein complex formed by β-catenin, axin, and adenomatous polyposis coli [13], which prevents GSK3-mediated β-catenin degradation and induces β-catenin–dependent gene transcription.

GSK3 phosphorylates the microtubule associated protein tau resulting in its hyperphosphorylation and subsequently paired helical filamentous tau (PHF-tau) formation, a key component of NFTs [14, 15]. In post-mitotic neurons, tau associates with microtubules and stabilizes their polymerization. In AD, increased GSK3β activity has been identified in postmortem AD brains [16]. Evidence indicates that the phosphorylated state of tau is closely associated with AD pathology [17] GSK3 phosphorylation sites on tau are believed to be abnormally phosphorylated in AD. Furthermore, $A\beta_{40,42}$ induces the formation of tau fibrils resembling PHF-tau in culture [18], and is also thought to increase GSK3 activity. PHF-tau is deposited as an insoluble misfolded aggregate protein in the somatodendritic compartment in postmortem AD brain tissue [19], and it is highly resistant to the action of phosphatases and proteases as it is often truncated at the C-terminal domain. These studies suggest that the inappropriate activation of GSK3 in the AD brain could play a role in the pathophysiology of PHF-tau formation.

GSK3α was reported to regulate Aβ production by positively modulating the γ-secretase complex [20], although this area of research is still being debated [21]. Inhibition of GSK3 activity with non-specific GSK3 inhibitors, such as lithium chloride and valproic acid in *in vitro* and in animal models of AD, has been shown to decrease Aβ production. More recently it was shown that specific inhibition of GSK3β, reduced BACE1-mediated cleavage of AβPP through a NF-κB signaling-mediated mechanism and consequently Aβ production by decreasing *BACE1* gene transcription and expression [22]. This is an important finding since the expression level and activity of BACE1 is reported to be elevated in AD patients [23]. Furthermore, inhibition of GSK3 signaling reduced Aβ deposition and neuritic plaque formation, and rescued memory deficits in a double transgenic AD mouse model [43]. Given the role of GSK3 in PHF-tau and the subsequent link to Aβ production, it appears that GSK3 could act as a common molecular link between amyloid plaque pathology and NFT pathology in AD. This is an area of intense research and such hypotheses will still require substantial validation, both pre-clinically and in the clinic.

Synaptic loss is one of the best correlates of cognitive deficits in AD [24]. It has been proposed that the mechanism allowing information storage in the brain involves changes in synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD). LTP inhibits GSK3β activity, and it is required for LTD, which indicates that the two phenomena are interrelated and that LTP regulates LTD [25]. In addition, within the synaptic compartment, tau and Presenilin 1 (a component of the γ-secretase complex that generates Aβ), may be additional targets for GSK3β. Specific overexpression of GSK3β in neurons causes a drastic decrease in postsynaptic density number and volume in hippocampal granule neurons [26], a phenomenon that could induce cog-

nitive impairment and altered LTP production [27, 28]. Since Aβ is thought to induce synaptic toxicity [29], and GSK3β activation is required for the pathological effect of Aβ on synaptic plasticity, it is tempting to speculate that GSK3β inhibitors could protect synapses from the deleterious effects of Aβ [30].

THE EARLY GSK3 DRUG DISCOVERY YEARS

During an era where genetics pointed to amyloid targets as the future for drug discovery research in the attempt to cure AD, several pharmaceutical companies including Zeneca, Astra, Mitsubishi Tanabe, Bristol Myers Squibb, and others were debating whether testing the alternate abnormal tau hyperphosphorylation hypothesis was worth an investment. Several academic researchers had proposed that tau, a microtubule associated protein present in axons, was hyperphosphorylated in AD brains and this aberrant hyperphosphorylated tau did not bind effectively to microtubules leading to destabilization. Consequently, axonal transport could no longer proceed efficiently resulting in synaptic and cognitive dysfunction [31–33]. Based on cellular, transgenic mouse data and expression and phosphorylation profiles in AD brain, GSK3 was implicated as a major kinase in the aberrant hyperphosphorylation of tau leading to NFTs in AD [34, 35]. Accordingly, inhibition of pre-tangle pathology via GSK3 inhibition would be expected to slow down the progression of NFT formation and neurodegeneration in AD. In addition, given the evidence that GSK3 inhibitors might be able to suppress the production of glucose by the liver, as well as enhance its conversion into glycogen, GSK3 was also an important target for type 2 diabetes.

While several companies successfully identified small molecule GSK3 inhibitors [36], in the following article, we focus specifically on the quest to identify suitable orally available GSK3 inhibitors as a disease modifying therapy for AD from AstraZeneca. Embarking on a drug discovery project targeting GSK3 in 1997, two independent high throughput screening (HTS) campaigns; one at Zeneca and the other at Astra resulted in the screening of approximately 2 million small molecule chemical compounds. Following the merger of AstraZeneca in 1999, the two geographically separated projects were combined into one project in Sweden in July, 2000. From the HTS assay, six chemical series were selected. These were the oxindolequinazolines, anilinoquinazolines, thiazoles, pyrimidines, thiazolidinediones, and pyrazines. The pyrazines and oxindolequinazolines were pursued based on druggability, intellectual property and the potential to expand and optimize structural activity relationships [37], and subsequently AZD2858 (Fig. 1a) from the

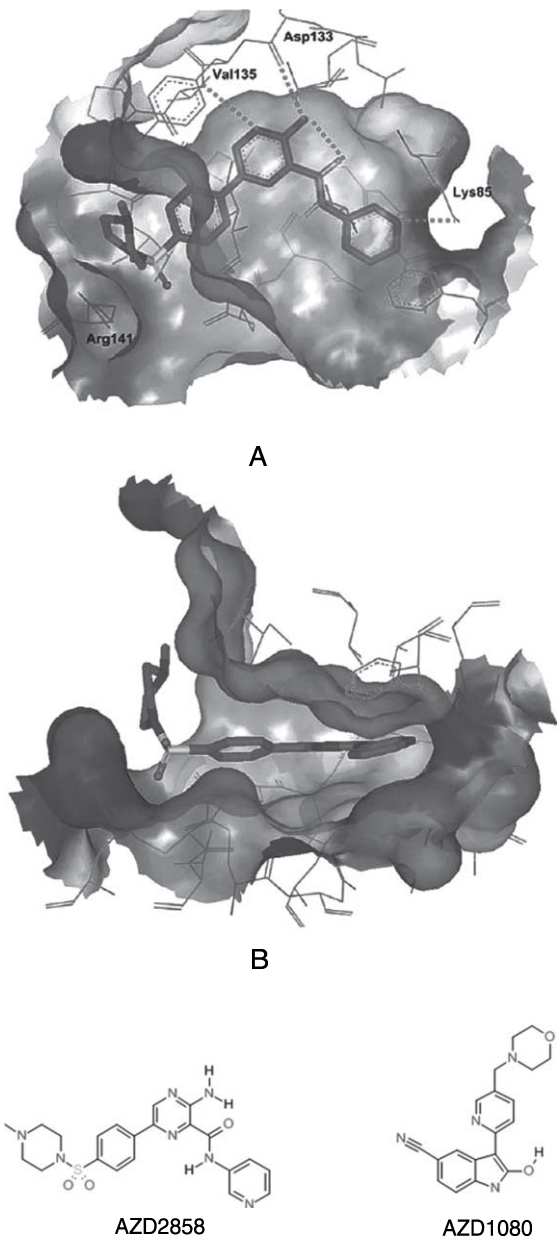

Fig. 1. X-ray crystal structure of GSK3 inhibitor AZD2858 (1a) that progressed through 28 day GLP toxicology studies (A: top view; B: side view) in the ATP pocket of GSK3β and the Phase I clinical candidate AZD1080 (1b).

pyrazine series progressed through two species 28-day GLP toxicology studies, and AZD1080 (Fig. 1b) from the oxindolequinazoline chemical series was progressed through Phase I clinical trials for AD [38]. Some of the other selective GSK3 inhibitors [39] were abandoned due to poor physico-chemical and drug-like properties.

The first clinical candidate AZD2858, a highly potent (4.8 nM) and selective orally bioactive brain penetrant GSK3 inhibitor, emerged in 2003 with excellent drug-like properties [37]. AZD2858 demonstrated dose-dependent inhibition of tau hyperphosphorylation in rodent brain hippocampus and inhibition of gliosis, a marker of neurodegeneration in a transgenic model overexpressing GSK3. Furthermore, AZD2858 inhibited PHF-tau formation in cellular models.

THE GLASS: HALF FULL OR HALF EMPTY?

Since GSK3 is a master switch regulating cell fate specification, ranging from proliferation and differentiation to regulation of glucose homeostasis, we anticipated some challenges in preclinical toxicology studies. In the regulatory IND enabling toxicology studies for AZD2858, we unexpectedly found that AZD2858 caused a rapid and robust increase in bone formation, including thickening trabeculae and osteoblast proliferation, in rats and dogs. The effect was only partially reversible four weeks after discontinuation of AZD2858 administration. These findings led to the hypothesis that inhibition of GSK3 affects the Wnt signaling pathway, thereby causing proliferation in bone tissue [40]. Consistent with this hypothesis, two key downstream targets of the Wnt signaling pathway, β-catenin and cyclin D1, were shown to be increased in the femur, tibia, and femoro-tibial joint in rats treated with a high dose of AZD2858. These serendipitous findings raised the possibility that AZD2858 could be potentially used for the treatment of bone disorders such as osteoporosis, fractures, and bone loss during myeloid leukemia. Further investigative studies clearly demonstrated that Wnt activation by inhibiting GSK3 caused β-catenin stabilization in mesenchymal stem cells and stimulated commitment towards osteoblasts and osteogenic mineralization *in vitro*. Furthermore, GSK3 inhibition by AZD2858 led to time- and dose-dependent increases in bone formation biomarkers, as well as reductions in resorption biomarkers, indicating increased bone anabolism and a reduced bone resorption. Surprisingly, the resulting bone formation appeared normal and was resilient, as analyzed by histomorphometry and biomechanical testing [41, 42]. In alignment with this, GSK3 inhibition was able to drive direct bone repair in an unstable fracture milieu in rats. Unfortunately, the severity of the findings in the preclinical toxicology studies were not conducive to further development of this compound for chronic treatment for an AD indication. There were numerous other target organs identified (including the bile duct, see below) and the compound was also shown to be genotoxic (clastogenic) which ultimately prevented further progression to the clinic. In parallel, AstraZeneca embarked a new project focused solely on trying to identify GSK3 inhibitors that could mimic the beneficial effects of AZD2858 in osteoporosis and bone disorders, potentially with local applications during dental or orthopedic surgery, similar to how bone morphogenic proteins are used.

A SECOND ATTEMPT

The toxicity findings raised the possibility that we needed to minimally engage with the GSK3 target *in vivo* and inhibit its activity by approximately 15–20% upon which a stoichiometric balance in tau phosphorylation would favor microtubule stabilization in neurons, but not drastically inhibit basal cellular functions of GSK3 elsewhere. This resulted in a quest to identify a GSK3 inhibitor from a distinct chemical series that was slightly less potent albeit highly brain penetrant. In 2004, a second candidate drug, AZD1080 was identified which selectively inhibited GSK3 (Ki 30 nM), and had a two-fold higher brain to plasma ratio than the previous clinical candidate. AZD1080, had a more suitable pharmacokinetic profile, and was from a separate chemical series (oxindolypyridine), and it was able to mitigate the toxicological effects on the musculoskeletal system that had been identified with AZD2858. AZD1080 specifically binds to and inhibits GSK3β within the ATP pocket of the catalytic domain (Fig. 1b). The crystal structure showed that the inhibitor binds through three hydrogen bonds, to the backbone atoms of Val-135 (both the amide N and the carbonyl O), a residue located in the hinge/linker region alongside of the ATP-binding pocket of the enzyme (Fig. 1b).

The importance of AZD1080 as a specific GSK3 inhibitor was validated by its capacity to interfere

with tau hyperphosphorylation *in vitro* and *in vivo*. In cells and in rodent brain following *in vivo* administration, AZD1080 is extremely efficacious at inhibiting tau phosphorylation, thereby addressing one of the fundamental tau hyperphosphorylation hypothesis in a preclinical setting. AZD1080 inhibited tau phosphorylation in cells over-expressing tau protein in a dose-dependent manner. AZD1080 is a brain permeable small molecule compound which has favorable oral bioavailability and pharmacokinetic (PK) profile. The PK-pharmacodynamic (PD) analysis suggests that peak exposure in the hippocampus is within 1 h while the effect on tau phosphorylation inhibition peaks at 6 h and the effect remains up to 24 h. This suggested that a shorter frequency of dosing regimen may be required in the clinic [38]. The advantage of such a finding is that larger safety margins could potentially be derived. The reason for this prolonged effect is unclear but could be attributed to the tight regulation of phosphorylation and dephosphorylation events on the tau protein.

GSK3 inhibitors have also been reported to influence cognitive processes under certain conditions, specifically in impaired systems. AZD1080 prevented the disruption of LTP induction caused by acute treatment with the NMDA receptor antagonist, MK-801 while AZD1080 applied to brain tissue slices obtained from non-compromised animals had no significant effect on LTP. These studies suggest that AZD1080 reverses synaptic plasticity and functional deficits in a dysfynctional neuronal system and the efficacious effect is likely because of modification of pathways downstream of GSK3 [38].

In the Phase I clinical trial, AZD1080 exposures that result in peripheral inhibition of GS activity in rodent PBMC correlated well with that observed in the Phase I multiple ascending dose study. These results demonstrated that for the first time a selective GSK3 inhibitor such as AZD1080 had the ability to inhibit the GSK3 enzyme in humans [38]. AZD1080 was well tolerated and demonstrated peripheral target engagement in Phase I clinical studies in healthy volunteers. Unfortunately, the histopathological changes observed in the gall bladder in the dog after chronic dosing, progressed to chronic cholecystitis without any exposure margins to what was believed to be clinically relevant doses, and the severity of these findings eventually forced us to abandon Phase II clinical trials.

For the subsequent programs, we tried yet another chemical series where we again identified biliary hyperplasia in rat as dose limiting toxicity, even though optimization efforts targeted increased fractional excretion through renal pathways. With the expectation that the margins to the biliary findings would erode, we explored two additional backup clinical candidates from separate chemical series, and initiated problem solving activities to understand drivers for the biliary toxicity. The physicochemical properties of these compounds in combination with the unique pharmacodynamics effects of GSK3 inhibition ultimately proved to be an unsurmountable hurdle with respect to preclinical toxicity.

DRUG TARGETING TO RELEVANT TISSUE: A NEW BEGINNING?

The ubiquitous expression of GSK3 in many tissues and organs has led to significant toxicological challenges following oral dosing and systemic exposure to the GSK3 inhibitors, ultimately leading to the failure of many GSK3 inhibitors either in the clinic or prior to their progression. However, GSK3 inhibitors are still currently pursued in clinical development. An example is Tideglusib (AMO-02), an inhibitor of GSK3β currently in clinical trials at AMO Pharma for the treatment of myotonic dystrophy. Nevertheless, as inhibition of GSK3 is such a powerful biological mechanism with the potential to positively impact many diseases, the challenge of how GSK3 inhibitors can be progressed as a potential therapeutic remains enigmatic.

One strategy to circumvent the issue of achieving therapeutic concentrations while limiting off-target effects is to specifically target the GSK3 inhibitor to the organ or even cells of interest. This can be achieved by, for example, conjugating a GSK3 inhibitor to a moiety which has a preference to bind to the target organ or cells, or encapsulating the GSK3 inhibitor in a drug delivery system which preferentially binds to the required organ or cells. This has recently been shown by conjugating a GSK3 inhibitor to an aspartic acid octapeptide which is known to adsorb to hydroxyapatite, the mineral portion of the bone, hence giving a molecular conjugate which targets bone fractures. In addition, the conjugated molecule assembles into micelles, which extends its circulation time while maintaining its fracture-targeting abilities. Another recent example has been reported[43] using a bone-targeted nanoparticle (NP) for delivery of a GSK3 inhibitor [43]. The NPs were functionalized with a peptide with high affinity for tartrate-resistant acid phosphatase (TRAP) to achieve

enhanced delivery to fractured bone. The TRAP binding peptide nano particles (TBP-NPs) showed improved accumulation at fractured bone as well as uptake in regenerative cell types such as mesenchymal stem cells and osteoblasts *in vivo* in mice. Thus, the approach of loading GSK3 inhibitor in TBP-NPs has the potential to enhance bone regeneration with improved therapeutic window using systemic delivery which is not currently possible.

Similar applications are being pursued in the field of metabolic diseases. Inhibition of GSK3, including specific GSK3β-cell knockouts, have been reported to increase proliferation of β-cells, leading to an increase in β-cell mass and improved glycemic control. One possibility is to direct a GSK3 inhibitor to the pancreatic β-cells via linking to an antibody, peptide, or a separate small molecule which targets a receptor or uptake mechanism, which is expressed specifically on β-cells (see Fig. 2). This strategy will allow the exploitation of the powerful and disease modifying action of GSK3 inhibition via a cell specific mechanism. Similar approaches have recently been explored to limit the systemic exposure of kinase inhibitors and thus improve their therapeutic index and/or restrict their activities to the cell type of interest. This should enable their application outside the oncology field. Novartis researchers have for example identified dual GSK3-DYRK1 inhibitors as inducers of pancreatic β-cell proliferation [44]. To assess the potential of this series to expand beta cell mass while maintaining beta cell function and to limit the risk of nonspecific proliferation of other cell type, they subsequently designed a delivery system by linking the dual kinase inhibitors to (+)-dihydrotetrabenazine derivatives [45]. (+)-Dihydrotetrabenazine is a ligand of vesicular monoamine transporter 2 and its derivative have been used as beta cell imaging agent. Although this conjugation strategy proved to positively impact biodistribution toward pancreatic accumulation, it negatively impacted the proliferative potency of the resulting conjugates thus requiring further optimization and underlining the requirement of highly potent cargos. Another recent example is the design of an antibody drug conjugate to deliver dasatinib, a very potent but unselective Bcr-Ab1 tyrosine kinase inhibitor and Src family kinase inhibitor, to human T lymphocytes by targeting the CXCR4 receptor [46]. These conjugates were shown to deliver dasatinib selectively to human T cells *in vitro* and to possess excellent *in vitro* immunosuppressive activity. These recent examples highlight the potential of using molecular conjugates to restrict the activity of potent kinase inhibitors to a specific target cell type and to improve their therapeutic index. An additional challenge when applying these molecular Trojan horse approaches to GSK3 inhibition for an AD purpose, would be the need for the conjugates to cross the blood-brain barrier (BBB) to achieve exposure. This is, however, an intense area of research, and screening for brain penetrant antibodies or optimization of fusion proteins for BBB penetration have been reported. Moreover, for less complex small molecule drug and peptide drug conjugates, the use of an additional component enabling BBB could be envisaged in the form of, for example, a Transferrin receptor or GLUT1 transporter ligand.

In addition, understanding and achieving kinase selectivity, for example, via allosteric inhibition or targeting unique kinase conformations, and both discovering and introducing polypharmacology into a molecule (for example, through the re-emergence of phenotypic screening), has advanced significantly in recent years. We believe that combining appropriate kinase selectivity, polypharmacology, and targeting of the inhibitor to the cell type of choice will provide novel treatment options for GSK3 inhibitors in AD, metabolic disease and beyond. The beneficial effects

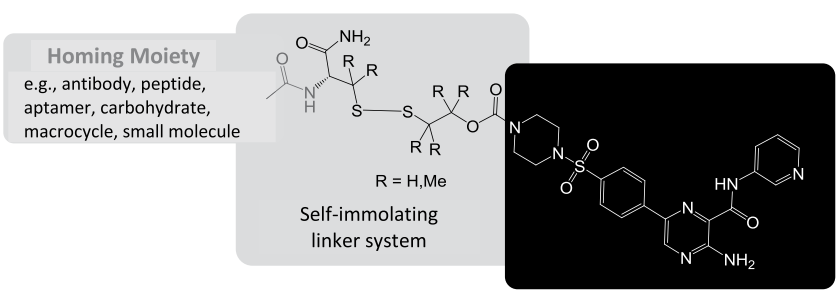

Fig. 2. Diagram showing drug targeting approach via homing moiety to the specific region (e.g., neurons, beta pancreatic cell), linker chemistry (green) and a GSK3 inhibitor (AZD2858)

of GSK3 inhibition has been demonstrated in a wide range of *in vitro*, cellular, and in proof-of-concept preclinical studies [4, 11, 25, 38, 47, 48]. However, this data needs to be translated both from an efficacy and safety standpoint to the clinic [49].

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9934).

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Tau Immunotherapies for Alzheimer's Disease and Related Tauopathies: Progress and Potential Pitfalls

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Abstract. Tau immunotherapies have now advanced from proof-of-concept studies to Phase II clinical trials. This review briefly outlines developments in the field and discusses how these therapies may work, which involves multiple variables that are connected in complex ways. These various factors are likely to define therapeutic success in humans and have not been thoroughly investigated, at least based on published reports.

Keywords: Alzheimer's disease, antibodies, clinical trials, immunotherapies, mechanisms, tau, tauopathy

INTRODUCTION

There are currently eight clinical trials ongoing on tau immunotherapies with several additional ones in late-stage preclinical development. However, the field is still in its infancy. Several therapeutic mechanisms may be involved and the importance of each one is not very clear. In addition, it is entirely unclear if the same pathway(s) of tau clearance will apply in human tauopathies. It is also quite possible that these promising therapies may work differently in different tauopathies, such as Alzheimer's disease (AD) versus progressive supranuclear palsy. The purpose of this review is to provide an up-to-date status of the field and to point out the various uncertainties and barriers to success, and how these may possibly be overcome.

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We published the first report showing the success of active tau immunization targeting the phosphoserine 396,404 region of the tau protein [1, 2]. This study was undertaken based on the success of targeting the amyloid-β (Aβ) peptide by similar means [3], and was originally laid out in an R01 application that was funded in 2001 and contained one aim to test this approach (Immune Therapy for AD Plaques and Tangles, NIH, 1R01AG020197, Principal Investigator: Einar M. Sigurdsson). A 30 amino acid peptide surrounding this region, Tau379-408[P-Ser396,404], was selected based on various computer algorithms that suggested that it was highly immunogenic, which we confirmed to be the case, and because of its prominent appearance in AD based on numerous prior publications. In the initial report, we showed that prophylactic immunizations in a mild alum adjuvant attenuated the development of brain tauopathy in JNPL3 mice, which have a familial tau mutation, P301L, and develop motor impairments as tau pathology advances in brain and spinal cord regions that influence movement. Less tau pathology

in the immunized mice was associated with functional improvements in motor tests, which further supported this approach. In this study, we also showed that antibodies isolated from a high titer mouse entered the brains of tauopathy mice but not wild-type mice following peripheral injection and could be found within neurons bound to pathological tau within 1 h after administration. We subsequently showed that this immunogen worked as well in a different tauopathy mouse model, htau/PS1, that does not have a tau mutation, and in which cognitive improvements were detected following the immunization regimen [4]. In later studies, we showed that tau antibodies could have the same effect [5, 6], and provided various insights into the possible mechanisms of clearance [7–19]. These studies have been confirmed and extended by various groups showing benefits of immune-targeting the pS396/404 tau epitope [2, 4, 6–9, 12, 20–27], and other tau epitopes [20, 23–25, 27–49], which as mentioned above has resulted in eight clinical trials (for a recent review of these trials, see [50]), with several more likely to be initiated in the near future. We have highlighted many of these studies in some details in various reviews over the years as they have been reported so it is not necessary to elaborate further on those particular aspects (for example, see [51–53]). Instead, I will go over some key points to consider as the field of tau immunotherapy matures.

HOW DOES IT WORK?

At the time of the R01 submission detailing this approach, very little was known about how various amyloids spread within or between organs. For tau in particular, there was no particular evidence for extracellular spread of tau between neurons. Hence, specifically targeting extracellular tau with antibodies could not be well justified, although we mentioned it as a target in more general terms [2, 54]. However, as detailed previously [2, 54], several papers had been reported over the years showing that antibodies could enter neurons, in particular under pathological conditions, for example to neutralize viruses. Various low affinity receptors that bind Fc portions of antibodies exist on neurons and are likely an important part of the immune system when need arises. Antibodies have to be able to travel to any site within the body to combat infections. Since tau is mostly found intracellularly, it could be reasoned that as long as the antibodies could get into the brain, as had been shown in the Aβ immunization studies, they would likely be able to enter neurons to neutralize and/or promote clearance of pathological tau, as we subsequently showed in our first report. We have previously elaborated on the intracellular mechanisms involved [54]. With regard to the extracellular component, it should be mentioned that in the prior decades, several studies by multiple investigators on different amyloids, other than tau, suggested that a spread through anatomically connected regions of the same organ or by various means between organs, was an inherent feature of these proteins and peptides that acquire the β-sheet conformation that defines amyloids. We have previously put forward this possibility in a brief review of these studies [55]. When extracellular spread of tau pathology was then shown to likely be an important part of tau pathogenesis (for review, see [56, 57]), pharmaceutical companies became particularly interested in targeting tau with antibodies as potential therapy for AD. Indeed, it seemed simpler and possibly safer than targeting tau intracellularly. The extracellular approach was more in line with targeting Aβ plaques, which had been shown to be successful, although those trials were starting to fail for other reasons. Those failures, and the well-known fact that the extent of tau pathology correlates better with the degree of dementia than Aβ burden, further shifted the early stage therapy studies to tau from Aβ. We have over the years in various venues suggested that both extra- and intracellular pathways are involved in antibody-mediated clearance of tau, and that the importance of each one may depend on several factors such as: 1) the tauopathy being targeted; 2) the stage of the disease, and; 3) the tau antibody (charge, epitope, isotype, affinity, whole versus fragment), which we discuss below.

1) The tauopathy being targeted

It has been shown by many groups that cerebrospinal fluid (CSF) levels of tau and pTyr181 tau increase in AD but not in various other tauopathies, compared to normal controls [58–60]. This consistent finding suggests that extracellular tau is unlikely to be an important component of tau pathogenesis in non-AD tauopathies. Hence, in those less common conditions, tau antibodies may need to work intracellularly to be effective. In this context, it is also important to note that recent mass spectroscopy studies show that most of CSF tau consists of tau fragments with ragged N- and C-termini that approximately consist of Tau150–250 [61, 62]. Although there should be less tau degradation in interstitial fluid, a gradient of such cleavage likely exists in

biological fluids that should influence antibody target engagement and clearance of pathological tau. Therefore, an antibody that targets tau outside the 150–250 region and only works extracellularly is likely to be less effective than: 1) such antibody that binds to tau intracellularly, where tau is less likely to have ragged termini; or 2) an extracellular antibody that targets the 150–250 region. Likewise, recent mass spectroscopy studies of total brain tau indicate the presence of a protease resistant core of varying lengths within residues 243–406 of the tau protein in AD and other tauopathies [63], suggesting at least a larger intracellular target pool for antibodies against this core.

2) The stage of the disease

It is likely that at least certain antibodies may work better or worse than others, depending on the stage of the tauopathy, with sub-variables including the brain region and the particular tauopathy. Analysis of brains from different stages of the disease has shown that particular tau epitopes appear at different stages of the disease. The presumed epitope profile of each individual may have to be considered when deciding whom to enroll in a clinical trial and eventually which tau antibody to describe for therapy. It is also likely that the accessible pool of intra- and extracellular tau changes during the course of the disease, which may then also influence the choice of antibody.

3) The tau antibody (charge, epitope, isotype, affinity, whole versus fragment)

With more publications on tau antibody therapies, it has become evident that various features of the antibody can greatly influence its mode of action and efficacy. The influence of some of these features was widely expected such as the epitope and isotype based on related studies targeting amyloid-β (Aβ) and to some extent other amyloids/protein aggregates. However, other features have been less studied and have sometimes led to surprising results. For example, we have shown that a low affinity antibody is more effective than a high affinity antibody against the same region [12]. However, this is a complex issue as these antibodies are likely conformational to some extent and recognize different tau species [12], which may have different degree of importance for tau pathogenesis. The potential influence of antibody size has not been well studied but we have shown that it greatly affects antibody uptake into neurons and not necessarily as you would expect [9, 11]. Finally, the importance of antibody charge for intracellular access has been well studied for potential cancer antibody therapies but not for tau antibodies or similar approaches targeting other amyloids. We have recently shown that antibody charge can robustly affect antibody uptake into neurons [19, 64], which may explain why some laboratories detect tau antibodies inside neurons [2, 7–9, 11, 12, 14, 39, 40, 65], whereas others do not for different antibodies [34, 41, 43]. This issue has particular importance for clinical trials because the humanized antibodies are likely to have a different charge than the mouse antibodies that they are based on. Therefore, the efficacy of the clinical candidate may be very different from its mouse counterpart, even though the binding site is the same. Other subtle structural changes associated with the humanization may also change the affinity profile of the antibody against different tau species. As mentioned above, higher affinity does not necessarily enhance efficacy and may actually have the opposite effect. It is also particularly difficult to anticipate how changes in affinity profile may influence efficacy because we do not really know which specific tau species are most pathogenic and/or most closely linked to functional deficits. It is also important to note that tau seeding or spreading may not necessarily be directly linked to tau toxicity. Both features of the disease are likely important for disease manifestation but may need to be tackled by different sets of antibodies, each of which may be more efficacious in certain tauopathies or at different stages of the disease.

As evident from this overview, the most variables rest within the antibody itself and these can be interdependent. Therefore, it is appropriate to discuss these in more detail, specifically for tau antibodies.

3a) Charge: We have published our preliminary findings within this important topic, with a more comprehensive manuscript under review [19, 64]. Specifically, we showed that tau antibodies against different tau epitopes (1B9: P-Thr212/P-Ser 214; 2C11: P-Ser262; Tau-5:210–244; and 4E6: P-Ser396/404) are taken up to a varying degree in primary neuronal cultures from tauopathy mice. This difference in uptake influences their efficacy and may be explained by charge differences as defined by their isoelectric point (IEP). Compared to the 4E6 antibody (IEP = 6.5), the other antibodies are taken up to a much lesser degree (1B9 IEP = 8.0; 2C11 IEP = 7.8; Tau-5 IEP = 5.1), indicating that a slightly acidic pH may be ideal for uptake, which decreases for more acidic or basic antibodies. To better compare how uptake affects efficacy, we then demonstrated that partial humanization of the 4E6 antibody, in which the Fc region and a part of the non-binding Fab region were replaced with a human scaffold, robustly shifted

the IEP from 6.5 to 9.6. This charge difference greatly reduced its neuronal uptake and efficacy. This latter experiment highlighted as well that humanization can dramatically alter the efficacy of the antibody, and that such antibodies should therefore be carefully studied before clinical trials to make sure that they will act as intended. We are not aware of other studies examining this for therapeutic tau antibodies but a report on single domain llama anti-tau antibody fragment as an imaging probe revealed a basic IEP (pI 9.5–10) for their diagnostic candidate [67]. It is likely that the potential effects of charge on neuronal uptake depends on the size of the molecule (antibody versus fragment).

3b) Epitope: The epitope that was a part of the immunogen in our original report has been most studied and repeatedly shown to be a good target [2, 4, 6–9, 12, 15, 20–27].

As mentioned above, an active vaccine, ACI-35, encompassing this epitope is now in clinical trials [68]. Targeting numerous other epitopes has been shown to be effective in several studies. These include non-phosphorylated [27, 30, 32, 41, 42, 46–48, 69, 70], phosphorylated [20, 24, 25, 27, 29, 35, 39, 43, 49], conformational/oligomeric [20, 31, 33, 34, 40, 44, 45], and a truncated epitope [66, 71]. Since these studies have various designs, they cannot be easily compared to identify the best epitopes to target. However, a few studies have examined side by side antibodies that bind to different tau epitopes but those antibodies differ in other ways such as in their affinity, isotype, and possibly charge as well [9, 12, 20, 23–25, 27, 31, 32].

3c) Isotype: This potentially important aspect has not been well studied, at least not publicly. If the antibody is acting extracellularly, one question that is being asked is if it should have an effector function or not, to facilitate microglial phagocytosis of the antibody-tau complex. Only one report has explored this issue on antibodies with an identical Fab binding portion, indicating that an effector function is not necessary for efficacy in clearing pathological tau [72]. A prior study comparing two antibodies that recognize a similar epitope (pSer404) with comparable affinity suggested that effector function is beneficial, with an IgG2aκ isotype being effective whereas an IgG1aκ was ineffective [23]. For tau antibodies acting intracellularly, isotype may influence receptor-mediated uptake which relies on the Fc portion [8, 40]. Antibodies of different isotypes may also differ in their charge which affects uptake. More studies are needed to clarify this variable but it is likely to be less important than for Aβ because the pool of extracellular tau is much smaller than for Aβ. Also, unlike Aβ, tau does not deposit in the vasculature, although it can be associated with it, which limits vascular side effects of the tau immunotherapy, which otherwise might be enhanced by microglial phagocytosis.

3d) Affinity: As mentioned above, we have recently reported that a low affinity antibody against the P-Ser396,404 region is effective in various culture and *in vivo* models, whereas a high affinity antibody against the same region is ineffective in the same models but more promising as a diagnostic imaging probe [12]. The antibodies are of the same isotype (IgG1k) but it should be emphasized that they differ not merely in affinity but also in their binding profile against various tau peptides and tau species from mice and humans. Although the profile differences are to some extent affinity related, they are also likely due to subtle differences in the exact epitope recognized although it is within the same region. A prior study showed that a low affinity antibody against a conformational epitope (MC1, aa7–9, and 312–342 [73]) was effective in a mouse tauopathy model, whereas a high affinity antibody, DA31, recognizing total tau (aa 150–190) was ineffective [31]. However, since the epitopes are very different, other factors may influence these findings. Most recently, our preliminary findings showed that partial humanization of a consistently effective mouse tau antibody, 4E6, strongly enhanced its affinity for various aggregated and insoluble tau species but rendered it ineffective in tauopathy culture models [19]. This lack of efficacy may be because it no longer bound to pathological tau in solution and likely in part because its neuronal uptake was very limited after the humanization. As mentioned above, such reduced uptake can be explained by its shift to a strongly positive charge from a slightly negative charge (see Antibody charge above).

3e) Size: Most of the antibodies that have been tested for efficacy are whole antibodies (150 kDa). When antibody fragments have been examined, they have been scFvs (25 Da) which differ in affinity as well and have not been compared to otherwise comparable whole antibodies containing the same CDR regions [74, 75]. Also, these two studies used ultrasound [74] or vectored expression [75] of the anti-tau scFvs, which further complicates direct comparison with standard whole antibody therapy design. Hence, it is difficult to say anything about how size influences efficacy. When we examined antibody uptake

in some detail, we compared whole antibodies, 4E6 and 6B2 against the P-Ser396/404 region, to their single Fab fragments (50 kDa) [9]. We expected it to be less because we had seen that about 80% of whole antibody uptake was Fc-mediated [8]. To our surprise, the percentage of neurons with antibody versus Fab signal increased from about 25% to about 70% in tauopathy brain slices and from less than 10% to about 60% in wild-type slices. The Fab fragment is taken up by bulk endocytosis, which is a much less prominent uptake pathway by whole antibodies (about 20% with 80% being receptor-mediated [8]). It is also a less specific pathway than receptor-mediated endocytosis that may explain the wild-type uptake. We have since reported in preliminary findings that tau antibodies in wild-type neurons are cleared much faster than in tauopathy neurons as detected by multiphoton imaging, presumably because the wild-type neurons do not have tau aggregates for the antibodies to bind to [18]. It has yet to be reported if there are efficacy differences between whole antibodies and their Fab fragments. Although the affinity should be the same, avidity of whole antibodies will be greater. Because of their smaller size, more of the fragment may enter the brain but its half-life is shorter and more of it may be lost via non-specific uptake and subsequent degradation. Even smaller antibody fragments, such as single domain antibodies (sdAbs; 13 kDa) that contain only a heavy chain variable region, should be explored for efficacy and diagnostic imaging potential.

TOXICITY CONCERNS

Most of the initial work on this important topic was conducted by the Rosenmann laboratory, which examined the feasibility of an active induction of an autoimmune disorder in mice by immunizing them with full length recombinant tau protein [76]. To promote this scenario, the mice received two very strong adjuvants, neither of which is approved for human use. The overall approach was indeed detrimental with the mice developing tauopathy and neurological deficits. Subsequently, her group showed that similar toxicity could be observed with repeated immunizations with phospho-peptide immunogens using the same strong adjuvants [77], but according to their prior work, not if fewer immunizations were used [28]. Others later reported no obvious side effect of mouse immunizations with full-length recombinant tau using a milder adjuvant [37]. We have previously mentioned in reviews our then unpublished observations of enhanced mortality in 3xTg mice and in wild-type mice of the same mixed strain background [51, 78], which has now been detailed in a publication [15]. Briefly, this particular mixed strain background led to a strong antibody response to Tau379–408[P-Ser396, 404], and substantial mortality after the fifth immunization. Surviving mice had sustained strong antibody titers until they were killed for analysis at an old age. Likewise, in a follow up study, mice of this strain background that received four immunizations, from 2–6 months of age, maintained strong antibody titers until the end of the study at 22 months of age, which resulted in not only less tau pathology but also near complete clearance of Aβ deposits [15]. Together, these findings suggest that patients receiving active tau immunizations should be carefully monitored to minimize unnecessary vaccine boosts that may have detrimental side effects. Immune response is haplotype-dependent and varies between individuals. Apparently, this has not been an issue in at least one of the two active tau immunization clinical trials, which administers a KLH-linked tau294–405 in alum adjuvant [79]. Information about the second trial has been limited [68].

None of the numerous passive tau antibody studies in tauopathy mice have reported any side effects. However, one study that administered a total tau antibody to Aβ plaque mice reported treatment induced mortality that should be examined further for that antibody in various tauopathy and wild-type mice [80]. In the clinical trials, there have not been any major side effects reported and those that started first have now advanced to Phase II. Human Phase I studies with one tau antibody were discontinued but apparently there were no safety or efficacy concerns, assuming that perhaps its half-life was too short.

A question that is often asked is if it is more likely that toxicity may be seen if a normal tau epitope is being targeted versus an epitope that is pathological or at least seen more prominently in the tauopathy than in healthy individuals. An advantage of targeting a normal tau epitope is that it is likely to be found as well in different forms of pathological tau, which then provides a greater pool to target. However, it should be kept in mind that these normal epitopes are often in the N-terminus, which appears to be cleaved away in many forms of tau (see Epitope section above). A short answer for antibodies is that targeting a normal tau epitope is unlikely to be very

toxic, as supported by several reports on such antibodies, because it is unlikely that the antibody will see much of normal tau, which is primarily located in the cytosol. Intracellularly, findings from us and others indicate that tau antibodies mostly interact with tau in the endosomal-lysosomal system and may facilitate their lysosomal degradation [2, 7–9, 11, 39, 40]. Presumably, within these vesicles, antibody binding to tau may loosen up tau assemblies and thereby allow better access of lysosomal enzymes. Some antibodies may leak into the cytosol from the endosomes or lysosomes via unknown mechanism, such as tau antibody 6B2 as we have reported [9]. Such leakage may potentially interfere with normal functions of tau, although we have not detected any side effects of that antibody. It was also recently reported that an interaction of a tau antibody with cytosolic Fc receptor, TRIM21, inhibits seeded tau aggregation [65]. This finding suggests that tau antibodies may also have a cytosolic target to facilitate proteosomal clearance of misfolded tau. Extracellularly, tau is detected under normal and pathological conditions but it is not clear if normal tau has any physiological extracellular function. If it does, clearing it extracellularly may be deleterious. It should also be kept in mind that other microtubule-associated proteins have similar functions as tau as reflected in the relatively normal phenotype of tau knockout mice [81]. Therefore, some antibody-mediated decrease in normal tau is unlikely to be detrimental per se, although there are always some concerns about possible development of an autoimmune disorder, in particular with active immunizations that are more irreversible than passive immunizations. Reduced tau levels have shown benefits in culture and mouse Aβ models [82, 83], further alleviating concerns, and the feasibility of interfering with tau expression is being pursued as a potential therapy for AD.

ONGOING CLINICAL TRIALS

There are currently eight clinical tau immunotherapy trials underway (Table 1). Two are on active- and six are on passive approaches.

The first trial uses a tau peptide encompassing amino acids 294-305 linked to KLH and administered in alum adjuvant in AD patients [36, 79]. This vaccine named AADvac-1, developed by Axon Neuroscience SE, is currently in Phase II [84].

The other active trial utilizes a phospho-serine 396,404 epitope that is administered in a liposome adjuvant in AD patients [22, 68]. That vaccine termed ACI-35, originally developed by AC Immune SA and then licensed to Janssen, is presently in Phase I [68].

The six passive trials consist of antibodies targeting:

1) tau8–19 in healthy subjects and PSP patients, that was developed by iPerian and subsequently by Bristol-Meyers Squibb and has now been licensed to Biogen [38, 85, 86]. Currently named BIIB092 (previously BMS-986168 and IPN007), it is in Phase I-II for PSP;
2) tau25–30 in AD (Phase II, [87]) and PSP (Phase II; [88]). It was developed by C2N Diagnostics, LLC (C2N-8E12; [32, 89]) and has been licensed to AbbVie (ABBV-8E12);
3) an unidentified epitope that may be phosphoserine 409 (RO7105705) in healthy subjects and AD patients [72, 90], and;
4) an unidentified epitope (LY3303560) in subjects that are healthy, or with mild cognitive

Table 1
Clinical trials on tau immunotherapy

	Tau Epitope	Subjects	Current Stage	Company
Active immunization				
AADvac-1	Tau294–305	AD	Phase II	Axon Neuroscience SE
ACI-35	P-Ser396,404	AD	Phase I	AC Immune SA – Janssen
Passive immunization				
BIIB092 (BMS-986168, IPN007	Tau8–19	Healthy, PSP	Phase I-II	Biogen (Bristol-Meyers Squibb; iPerian)
ABBV-8E12 (CN2-8E12)	Tau25–30	AD, PSP	Phase II	AbbVie (C2N Diagnostics)
RO7105705	P-Ser409?	Healthy, AD		AC Immune SA – Genentech – F. Hoffman La Roche AG
LY3303560	Conformational (7–9, 312–342)	Healthy, MCI, AD	Phase I	Eli Lilly
RG7345, RO6926496	P-Ser422	Healthy	Phase I - discontinued	F. Hoffman La Roche AG
JNJ-63733657	Middle region	Healthy, AD	Phase I	Janssen
UCB0107	235-246	Healthy	Phase I	UCB Biopharma

AD, Alzheimer's disease; PSP, progressive supranuclear palsy; MCI, mild cognitive impairment.

impairments or AD (Phase I, [91, 92]) that is likely a humanized form of the conformational antibody MC1 [73, 93], which as mentioned above has been effective in different mouse studies [20, 31].

In addition, one trial examining an antibody against phospho-serine 422 (RG7345, RO6926496) was discontinued in its early Phase I stage in healthy subjects that are unlikely to have that epitope, presumably because of a poor pharmacokinetic profile.

Several other active and passive immunotherapies are in preclinical studies as detailed above and at least some of these are in clinical development and will enter clinical trials in the near future. As the ongoing trials are in their early stages, not much has been published about their progress. However, the fact that many have advanced to Phase II indicates that the therapies are not toxic and most likely some degree of target engagement has been observed.

FUTURE DIRECTIONS

It will likely be several years before we know if tau immunotherapies will be efficacious to slow the progression of tauopathies. Assuming that at least some of them will work, it is then likely that more resources will be put into developing active tau immunizations. Those are inherently riskier than passive immunotherapies because of the potential for immune response related side effects, in particular unwanted T-cell activation that may be difficult to control. One way to minimize such adverse reactions would be to tailor the vaccine to individual recipients. For example, by considering the haplotype of the patient, a tau immunogen can be selected that is likely to provide the desired antibody response while minimizing T-cell epitopes that could be detrimental to that particular person. An appropriate antibody response could be further modulated with different adjuvants to ensure sufficient but not too strong immune response to the vaccine. Once sufficient antibody titer is achieved, period boosts could be provided to maintain it. Overall, that approach would be much less expensive than monthly antibody injections and therefore larger populations could be treated. Eventually, after establishing sufficient safety profile, active immunization could possibly be used prophylactically. That will likely first be tested in individuals with familial mutations that will cause AD or other tauopathies, and subsequently in persons that, for known or unknown reasons, have an increased risk of developing a tauopathy.

Advances in tau brain imaging have now resulted in several promising β-sheet dye compounds that appear to be selective for tau aggregates, although non-specific binding has now been reported for some of them and their use discontinued [94, 95]. Also, these probes are not good at detecting non-AD tauopathies, suggesting some structural differences in the tau lesions [94, 95]. Antibody fragments should be more specific and, if they can be delivered to the brain in sufficient amounts for PET detection, may allow documenting the epitope profile of the individual that could result in a more personalized immunotherapy, targeting those specific epitopes. Promising findings have been reported on this approach by us and others, supporting its further development [11, 67].

CONCLUSIONS

Following our report in 2007 showing the feasibility of tau immunotherapies, findings from multiple groups have confirmed and extended this approach, which has resulted in several clinical trials that have now advanced into Phase II. It will be several years until we know if any of these will be effective. Some may fail for various reasons, some of which are outlined above. Hopefully many others will show disease modifying benefits, which should then spark the initiation of additional trials that may include combination therapies at the earliest stages of the disease that could be guided by improvements in tau imaging probes.

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The Amyloid-β Oligomer Hypothesis: Beginning of the Third Decade

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Abstract. The amyloid-β oligomer (AβO) hypothesis was introduced in 1998. It proposed that the brain damage leading to Alzheimer's disease (AD) was instigated by soluble, ligand-like AβOs. This hypothesis was based on the discovery that fibril-free synthetic preparations of AβOs were potent CNS neurotoxins that rapidly inhibited long-term potentiation and, with time, caused selective nerve cell death (Lambert et al., 1998). The mechanism was attributed to disrupted signaling involving the tyrosine-protein kinase Fyn, mediated by an unknown toxin receptor. Over 4,000 articles concerning AβOs have been published since then, including more than 400 reviews. AβOs have been shown to accumulate in an AD-dependent manner in human and animal model brain tissue and, experimentally, to impair learning and memory and instigate major facets of AD neuropathology, including tau pathology, synapse deterioration and loss, inflammation, and oxidative damage. As reviewed by Hayden and Teplow in 2013, the AβO hypothesis "has all but supplanted the amyloid cascade." Despite the emerging understanding of the role played by AβOs in AD pathogenesis, AβOs have not yet received the clinical attention given to amyloid plaques, which have been at the core of major attempts at therapeutics and diagnostics but are no longer regarded as the most pathogenic form of Aβ. However, if the momentum of AβO research continues, particularly efforts to elucidate key aspects of structure, a clear path to a successful disease modifying therapy can be envisioned. Ensuring that lessons learned from recent, late-stage clinical failures are applied appropriately throughout therapeutic development will further enable the likelihood of a successful therapy in the near-term.

Keywords: Alzheimer's disease, amyloid-β peptide, diagnostics, etiology, model systems, oligomers, prions, receptors, structure-function, tau, therapeutics

Abbreviations: α7nAChR, alpha 7-nicotinic acetylcholine receptor; 5XFAD, transgenic mouse model of AD carrying 5 AD-related familial mutations; A11, amyloid oligomer polyclonal antibody; Aβ, Amyloid β peptide; Aβ40, Amyloid β peptide 1–40 sequence; Aβ42, Amyloid β peptide 1–42 sequence; Aβ43, Amyloid β peptide 1–43 sequence; AβOs, Aβ oligomers; AD, Alzheimer's disease; AkT, Protein Kinase B; ALS, Amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor; APOE, Apolipoprotein E gene; ApoE, Apolipoprotein E; APP, Amyloid precursor protein; AFM, atomic force microscopy; BACE, β-secretase; Ca^{++}, calcium ion; CaMKII, Ca^{++}/calmodulin-dependent protein kinase II; cDNA, complementary DNA; CNS, central nervous system; CSF, cerebrospinal fluid; CT, cortex; CTAD, Clinical Trials on Alzheimer's Disease; CTE, chronic traumatic encephalopathy; DHA, docosahexaenoic acid; DPP4, dipeptidyl peptidase 4; EphB2, Ephrin type B receptor 2; EphA4, Ephrin type A receptor 4; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; Fab, fragment antigen-binding; fAD, Familial Alzheimer's disease; FAK, focal adhesion kinase; FcγRIIb, Immunoglobulin gamma Fc region receptor II-b; FPR2, N-formyl peptide receptor 2; Fyn, tyrosine-protein kinase Fyn; GSK3β, glycogen synthase kinase 3 β; GTPase Drp-1, GTPase dynamin-related protein 1; HDAC6, histone deacetylase 6; HMW, high molecular weight; HP, hippocampus; i.c.v., intracerebroventricular; IGF-1, insulin-like growth factor 1; iPSC, induced pluripotent stem cells; IR, insulin receptor; IRS-1, insulin receptor substrate 1; kDa, kilodalton;

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LilRb2, leukocyte immunoglobulin-like receptor subfamily B member 2; LMW, low molecular weight; LRP-1, lipoprotein receptor; LTD, long-term depression; LTP, long-term potentiation; MCI, mild cognitive impairment; mGluR5, metabotropic glutamate receptor 5; MRI, magnetic resonance imaging; NADPH, nicotinamide adenine dinucleotide phosphate; NHPs, non-human primates; NKAα3, Na^+/K^+ ATPase alpha 3 subunit; nM, nanomolar; NMDARs, N-methyl-D-Aspartate receptors; NO, nitric oxide; NU4, AβO-selective mouse monoclonal antibody; N-VSCCs, N-type voltage-sensitive calcium channels; OC, anti-amyloid fibril antibody; p38 MAPK, p38 mitogen-activated protein kinases; p75NTR, p75 neutrophin receptor; pE, pyroglutamylated; PET, positron emission tomography; PICUP, photo-induced crosslinking of unmodified proteins; POPC/POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS); PPAR-γ, peroxisome proliferator-activated receptor gamma; PrPc, cellular prion protein; PS1, presenilin-1; PSEN1, presenilin-1 gene; pTau, phosphorylated tau; Pyk2, protein tyrosine kinase 2; RAGE, receptor for advanced glycation endproducts; ROS, Reactive Oxygen Species; sAD, Sporadic Alzheimer's disease; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SEM, standard error of the mean; Sigma-2/PGRMC1, Sigma-2 receptor/progesterone receptor membrane component 1; TBI, traumatic brain injury; TNF, tumor necrosis factor; ThioS, Thioflavin S; Tg, transgenic; TRPM2, transient receptor potential melastatin family subtype 2; VEGF-A, vascular endothelial growth factor A.

INTRODUCTION TO THE AβO HYPOTHESIS

The transition from the amyloid cascade to the AβO hypothesis

The detection of amyloid-β oligomers (AβOs) in human brain parenchyma and vasculature was first reported while the original amyloid cascade hypothesis was being introduced and developed [1–3]. At the time, AβOs were regarded as intermediates *en route* to generation of amyloid plaques, which were believed to be the pathogenic form of Aβ.

Today, AβOs are widely regarded as the most toxic and pathogenic form of Aβ (Fig. 1) [4, 5]. AβOs show an Alzheimer's disease (AD)-dependent presence in humans and animal models [1, 6–13], and their buildup occurs early, before plaques, evidenced by both immunochemistry [14] and immunohistochemistry [15, 16]. In support of a toxic role for AβOs and not plaques, the Osaka familial AD mutation of Aβ (APP E693Δ) shows extremely low levels of senile plaques [17–21] despite severe cognitive impairment [17, 20], while cerebrospinal fluid (CSF) manifests low levels of overall Aβ, but elevated levels of AβOs [22]. Transgenic (Tg) mice carrying this mutation [19], or a closely related one [23], likewise manifest AβOs and other major forms of AD neuropathology but not plaques. Although historically AD has been defined as dementia with plaques and tangles, replacing plaques with AβOs in this definition may be closer to the pathogenic mechanism.

Synthetic AβOs can assemble at very low concentrations of $Aβ_{42}$ monomer, in harmony with pre-plaque buildup in brain tissue [24, 25]. *In vitro*, AβOs form within minutes from low nanomolar concentrations of monomeric $Aβ_{42}$ [26, 27]. Because Aβ has been found to aggregate in sodium dodecyl sulfate (SDS) [28], some investigators have concluded that the quickly forming AβOs seen in SDS-PAGE are experimental artifacts [29, 30]. However, under full denaturing conditions, SDS-PAGE experiments show monomeric Aβ in the complete absence of AβOs [31]. AβOs can be observed, moreover, in the absence of SDS by atomic force microscopy (AFM) and by size exclusion chromatography [26, 31]. Evidence for structural homology between certain forms of synthetic and brain-derived AβOs has been presented [6]; this is discussed further below.

Besides their presence in brain, AβOs show an AD-dependent buildup in human CSF. An ultrasensitive assay, known as the BioBarcode, which is capable of attomolar measurements, showed median levels of AβOs in CSF from AD patients to be 30-fold higher than from non-demented individuals [32]. This elevation is opposite to the AD-dependent change measured in monomeric Aβ levels, which decrease rather than increase [33]. Levels are so low, however, that for most assays, comparisons of CSF AβO levels are not feasible [12, 32, 34, 35]. Ultrasensitive assays for AβOs in CSF, however, are extremely lengthy and difficult, and their lack of precision requires multiple runs to provide a reliable measurement. These are all factors that preclude their adaptation for the clinic.

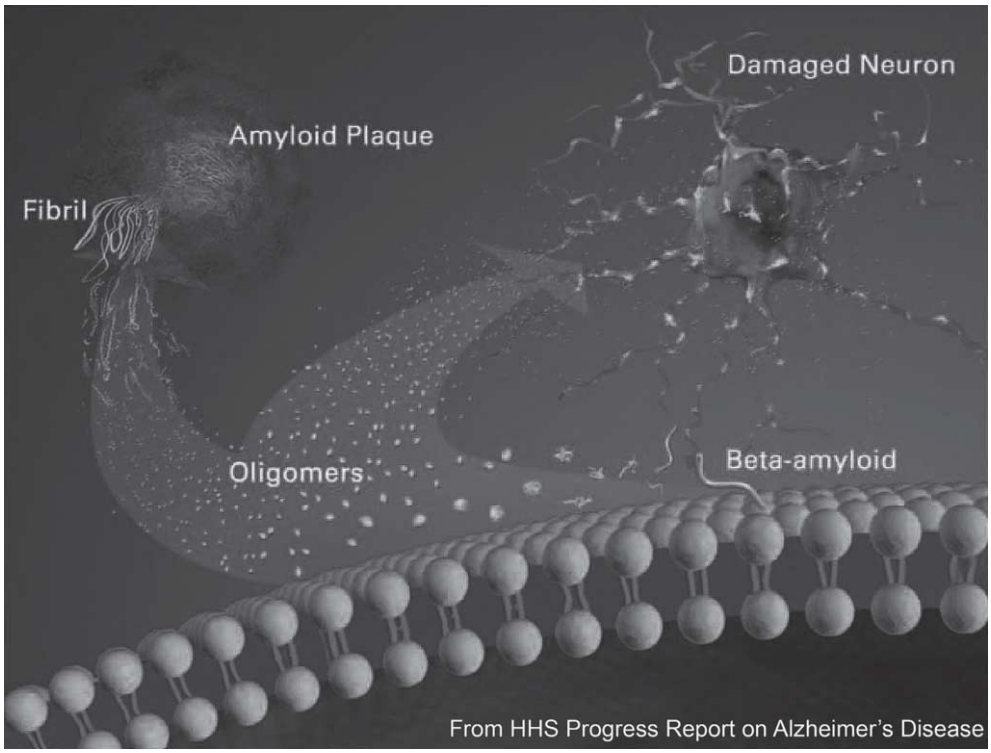

Fig. 1. **AβOs, not Aβ monomers or fibrils, instigate the neuron damage leading to dementia.** Following cleavage from the membrane, Aβ peptides aggregate to form AβOs, some of which further aggregate to fibrils and some of which instigate the neuron damage leading to dementia. Reprinted with Jannis Productions permissions from the "Progress Report on Alzheimer's Disease 2004-2005" (ed. AB Rodgers), NIH Publication Number: 05-5724. Digital images produced by Stacy Jannis and Rebekah Fredenburg of Jannis Productions [455].

There is extensive evidence that elevated AβO levels in the brain has pathogenic consequences, with results coming from behavioral, neuropathological, and cell biological studies, as discussed below. Memory performance is lost when small quantities of AβOs are injected into the intracerebral ventricle (i.c.v.) of non-Tg animals [36–39]. Long-term potentiation (LTP) and long-term depression, the electrophysiological underpinnings of memory formation, are disrupted by AβOs both *ex vivo* and *in vivo* [26, 36, 37, 40, 41]. Synthetic and brain-derived AβOs both exhibit these characteristics. In addition to their cognitive impact, exogenous AβOs instigate multiple facets of AD-neuropathology in culture and animal models, including non-human primates (NHPs) [42–46]. If one assumes an AβO molecular weight in aqueous solution of ∼100 kDa (see below), these effects are elicited at sub-nanomolar AβO concentrations [26, 47–50]. Overall, AβOs have been found to instigate tau pathology [19, 51, 52], loss of neuronal polarity [53–55], impairment of axonal transport [56–58], deterioration of synapses [47, 55], oxidative stress [59–62], endoplasmic reticulum (ER) stress [18, 63, 64], insulin resistance [48, 65–67], neuroinflammation [19, 49, 68, 69], cholinergic impairment [70, 71], loss of trophic factors [45, 72–75], epigenetic changes [74, 76–80], ectopic mitosis [81–83], and selective nerve cell death [26, 84]. A complicating factor is that these various responses were obtained under widely divergent conditions, with different disease models, time-scales, doses, and AβO preparations. Nonetheless, the collective body of evidence offers strong support for a mechanism in which AD neuropathology and cognitive loss are the consequences of the cellular damage instigated by AβOs (Fig. 2).

The evidence is strong that AβOs are manifested in AD brain. Experiments in animal models strongly suggest, furthermore, that AβOs are both necessary and sufficient for dementia. Sufficiency, at least vis-á-vis amyloid plaques, is indicated by instances of AD without senile plaque pathology. Highly demented individuals with the Osaka mutation (APP E693Δ) manifest AβOs (and other facets of AD pathology)

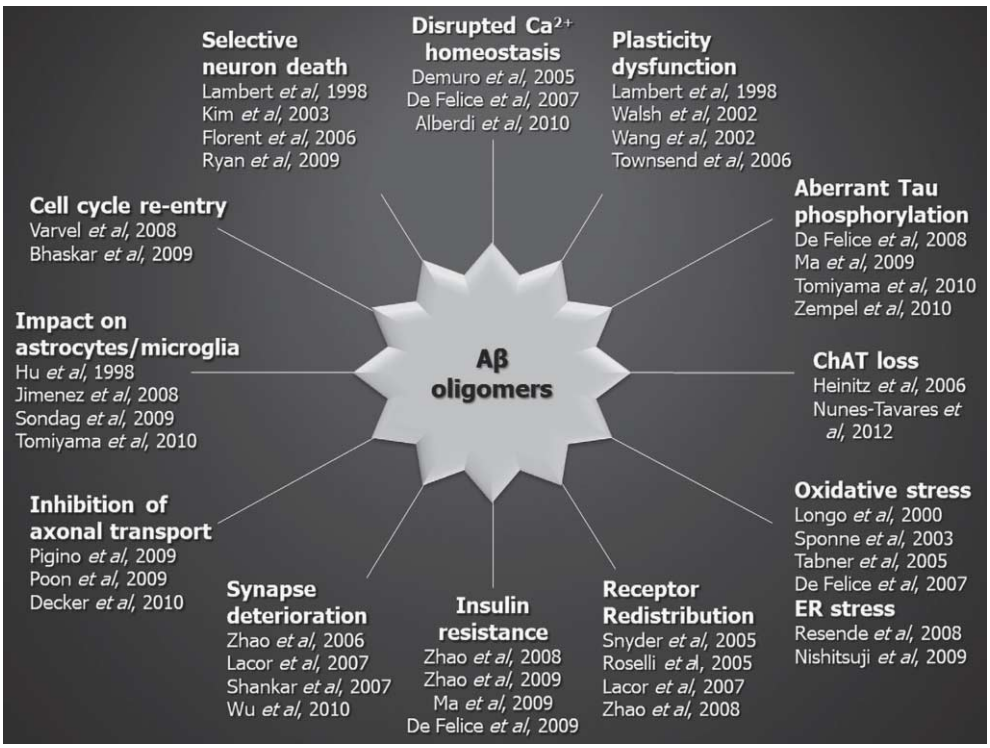

Fig. 2. AβOs instigate multiple facets of AD-neuropathology. Observed in various culture and animal models. Reprinted by permission from Springer Nature: *Acta Neuropathol*, 129(2): 183-206, "Amyloid beta oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis" by Viola KL and Klein WL. Copyright 2015 Springer Nature [200].

in the absence of senile plaques [17–21]. This has been experimentally recapitulated in a Tg mouse model harboring this mutation [19]. In addition, Tg mice expressing a different mutation in the same APP residue (Dutch APP E693Q) also exhibit AβO accumulation and altered synaptic structure without plaques [23, 85]. The sufficiency of AβOs for pathogenesis was first indicated in an APP mouse (Indiana APP mutation V717F; outside of Aβ42 sequence) that showed synapse loss despite absence of plaques [86]. In addition, a Tg rat expressing the Indiana mutation also shows pre-plaque AβO-associated cognitive impairment [87]. A later study comparing Tg strains indicated in fact that elevated levels of amyloid plaques likely protected against pathogenic AβO buildup [88].

Direct evidence that AβOs are necessary for dementia comes from experiments using AβO-selective antibodies. Such antibodies were first shown to protect cell models against the damage caused by exogenous AβOs [51, 89, 90]. When administered to various Tg AD mice, the antibodies prevent AD-like pathology and rescue memory performance [89–94]. New data from our group indicates that a single injection of an AβO-selective antibody (30 μg) can suffice to rescue memory performance in 6-7-month-old Tg 5xFAD mice for at least 40 days (Fig. 3; Bicca and Klein, unpublished data). AβOs and plaques in these mice begin to accumulate extensively around 2 months of age [11, 92, 95]. The new data are in harmony with previous evidence that an AβO-selective antibody can reach the parenchyma and engage AβOs [96], but not Thioflavin S (ThioS)-positive amyloid plaques, when injected into 5xFAD mice.

The large body of evidence that AβOs are both necessary and sufficient to trigger AD-associated memory malfunction and neurodegeneration, coupled with the extensive portfolio of documented AβO-triggered cellular and behavioral effects, sets the stage for new AD therapeutic approaches targeting AβOs. As the third decade of the AβO hypothesis begins, the biggest challenge is to mobilize a clinical trial that will validate or invalidate the hypothesis. While "Aβ dyshomeostasis has emerged as the most extensively validated and compelling therapeutic target" [5], the past development of Aβ-based therapeutics has largely concerned plaque elimination, ignoring AβOs. However, the link between

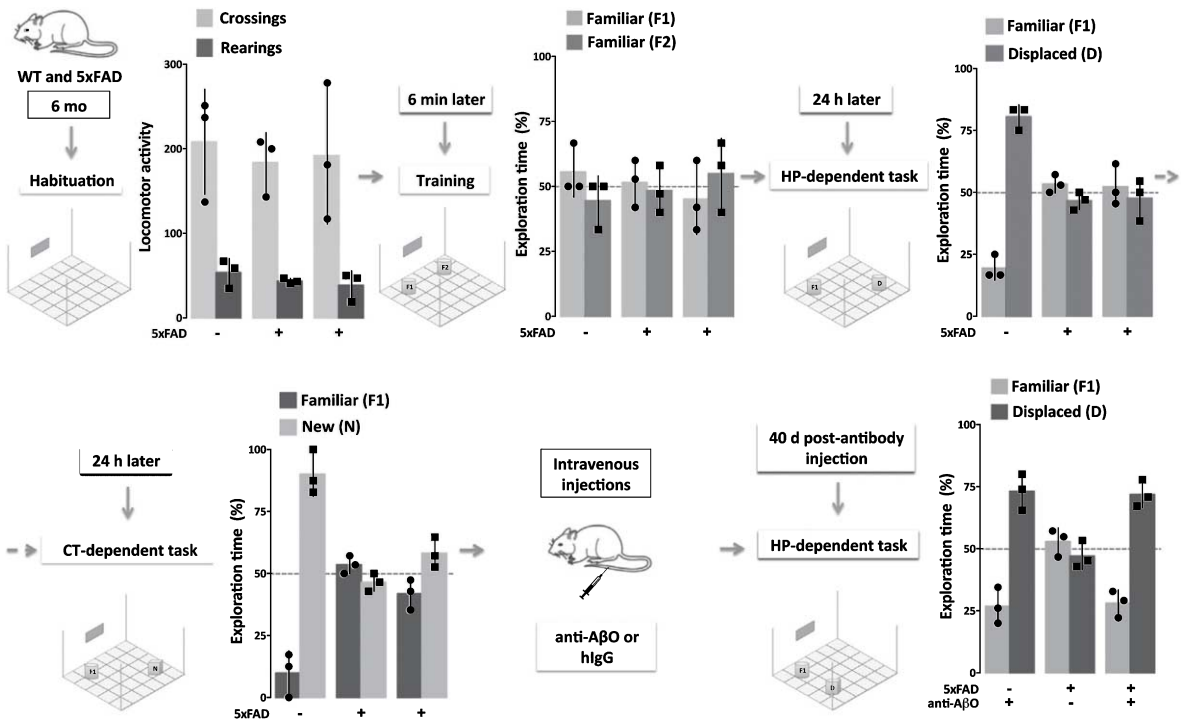

Fig. 3. Single injection (30 μg) of an AβO-specific antibody ameliorates cognitive deficits in AD mice for at least 40 days. 5xFAD Tg mice and their wild-type (WT) littermates (6 months of age) were evaluated by Object Recognition Tasks before and after (40 days) a single injection (30 μg) of a humanized AβO-specific antibody (anti-AβO) or non-specific human IgG (hIgG). First, locomotor activity was assessed while mice were allowed to habituate to the testing field (Habituation). Assessments were the number of times the mice crossed grids in the field (Crossings, light gray) and the number of times mice put their hind paws on the walls of the field (Rearings, purple), with no differences between WT and 5xFAD mice. Next, the test objects (F1 and F2) were introduced to the mice in the Training session. All mice showed normal exploratory behavior, defined by 50% exploration of each object, as both objects are equal and new to the mice. The ability of mice to remember object placement was then tested 24 hours after the Training session in a hippocampal (HP)-dependent task. Another 24 hours later, the ability of mice to remember the object was tested in a cortical (CT)-dependent task. Only the WT mice were able to recognize the familiar object (F1) from the Training session, as evidenced by >50% exploration of the displaced (D, pink) or new (N, light blue) object. The 5xFAD mice failed to recognize F1 in both tasks. When re-evaluated 40 days post-antibody injection in a HP-dependent task, only the 5xFAD mice that received the AβO antibody recovered their ability to recognize object F1. These data support the hypothesis that AβOs induce memory dysfunction in AD (Bicca and Klein, unpublished).

plaques and cognitive dysfunction has been tenuous for decades [97–99], and no Aβ-directed therapeutic has yet reached a clinical efficacy endpoint [100–103]. In a potential turning point, an antibody that can engage AβOs, Aducanumab, has recently shown modest therapeutic benefit in early clinical trials [104, 105]. A potential limitation of Aducanumab is that it lacks stringent selectivity for AβOs. Off-target engagement with senile plaques likely accounts for the high dosage-requirement found in trials. Antibodies are needed that target only the most pathogenic configurations of Aβ, i.e., AβOs. Such antibodies will be optimized by a better understanding of AβO structure-toxicity relationships [101, 106–108],

Besides development of AβO-specific antibodies [89, 101, 109], other tactics are likely to improve the prospects of Aβ-directed therapies. Such tactics may be earlier intervention within the disease continuum and better criteria for patient selection [5, 108] and better biomarkers for monitoring of investigational new drugs [103], including inflammation markers to better predict complications [106]. Furthermore, multi-factorial therapies may be needed [106]. Although it has been suggested that Aβ-targeting therapies may only be beneficial in prodromal individuals [110], if AβOs play a role in disease progression, e.g., through promoting propagation of tau pathology (below), there may be a meaningful chance that AβO-immunotherapy would be beneficial even after AD onset. Overall, there is an important call for more rigor in preclinical development. At each phase of the drug discovery process

for Aβ-targeting therapies, it has been possible to find significant gaps in data [102]. Target engagement, e.g., was not established for the majority of therapeutic agents analyzed [102, 107]. Furthermore, compounds have been moved into phase III trials on the basis of very limited data [111], premature moves that have had a tendency to poison the well. Consequently, the discouraging track record of Aβ-directed drugs has provided significant impetus to point new drug discovery efforts toward non-Aβ targets, despite the preponderance of evidence that AβOs are the culpable AD neurotoxins [112–114].

STATUS OF THE FIELD

In lieu of clinical efforts based on the AβO hypothesis, there nonetheless have been substantial developments in the last five years regarding more fundamental issues. Of the more than 4,000 publications on Aβ oligomers or oligomeric Aβ, about half were published in the last five years. These fundamental developments regarding AβO pathogenicity are just beginning to be tested clinically and we predict that they will set the stage for therapeutic success. This section will consider major developments regarding: 1) species of AβOs, their assembly, and relation to amyloid plaques, and emerging insights into how to approach molecular structure; 2) mechanisms of how AβOs initiate their impact on neuronal function and structure; 3) downstream pathways resulting in neural damage, and 4) multicellular interactions contributing to AβO pathogenicity.

A multitude of AβO species or just two?

One of the biggest knowledge gaps currently facing the field is the precise identity of the most toxic AβO structures [5, 29, 101, 106, 107, 115–117]. Without this knowledge, it is impossible to know if Aβ-directed therapeutics are engaging the correct target. Characterization of AβO structure has been hindered by AβO metastability and heterogeneity [116, 117]. Consequently, a multitude of AβO species have been identified in the literature [117]. It is not clear which of these AβO species are AD-relevant and which are experimental artifacts. One possibility is that there exist a multitude of pathogenically-relevant AβO species in the AD brain and that their high number correlates with the myriad Aβ-associated toxic pathways identified in the literature [29, 101, 106, 115]. Another possibility is that there are only a few discrete AD-relevant species, and the majority identified in the literature are merely artifacts induced by non-physiological experimental conditions [107, 116, 117]. As stated by Benilova and colleagues, "The lack of a common, agreed-upon experimental description of the toxic Aβ oligomer makes interpretation and direct comparison of data between different research groups impossible [117]."

Some patterns regarding AβO structure-toxicity relationships are, however, already emerging in the literature. For instance, it appears as if AβOs, whether produced *in vitro* or present in the brain of animal models or AD patients, can be divided into toxic and non-toxic sub-populations based on simple aspects of their quaternary structure, molecular weight and antibody reactivity, as well as their relationship to amyloid plaques. The toxic AβO species appear to be greater than 50 kDa [16, 55, 118], reactive with the anti-amyloid oligomer antibody A11 [119] and the anti-AβO antibody NU4 [120], and unrelated to amyloid plaques (Fig. 4) [118, 119]. On the other hand, the non-toxic AβO species appear to be less than 50 kDa [16, 55, 118], reactive with the anti-fibril antibody OC [119], and related to amyloid plaques temporally, spatially, and structurally [118, 119]. In addition to their convenient immuno-identification, they also can be separated *in vitro* by size exclusion chromatography [31] or ultrafiltration with a 50 kDa molecular weight cutoff [16, 55, 118]. These populations have been referred to in the literature, respectively, as "peak 1" and "peak 2" [31], high molecular weight (HMW) and low molecular weight (LMW) [16, 55, 115, 118], and "type 1" and "type 2" [119]. Myriad evidence supports a toxic role for type 1 AβOs. *In vitro*, they bind cultured synapses (Fig. 5) [16, 55, 118], inducing production of reactive oxygen species (ROS) [39], while type 2 AβOs cannot. Both species have been implicated in binding PrPc [121–123]. *In vivo*, type 1 AβOs disrupt memory function [39, 119, 120]. Type 2 AβOs have been found not to be associated with memory dysfunction [119, 120], although in one study, they were [39]. HMW, type 1 AβOs appear to be most prominent AβOs in the AD brain under native conditions [124–126]. The differential toxic impacts of LMW and HMW AβO species has been recently reviewed by Ferreira and colleagues [115].

One specific type 1 AβO has been identified, the 56 kDa SDS-stable species sometimes referred to as Aβ*56 [44]. Aβ*56 was first identified as a prominent AβO in AD brain [6] and Tg2576 mice [44]

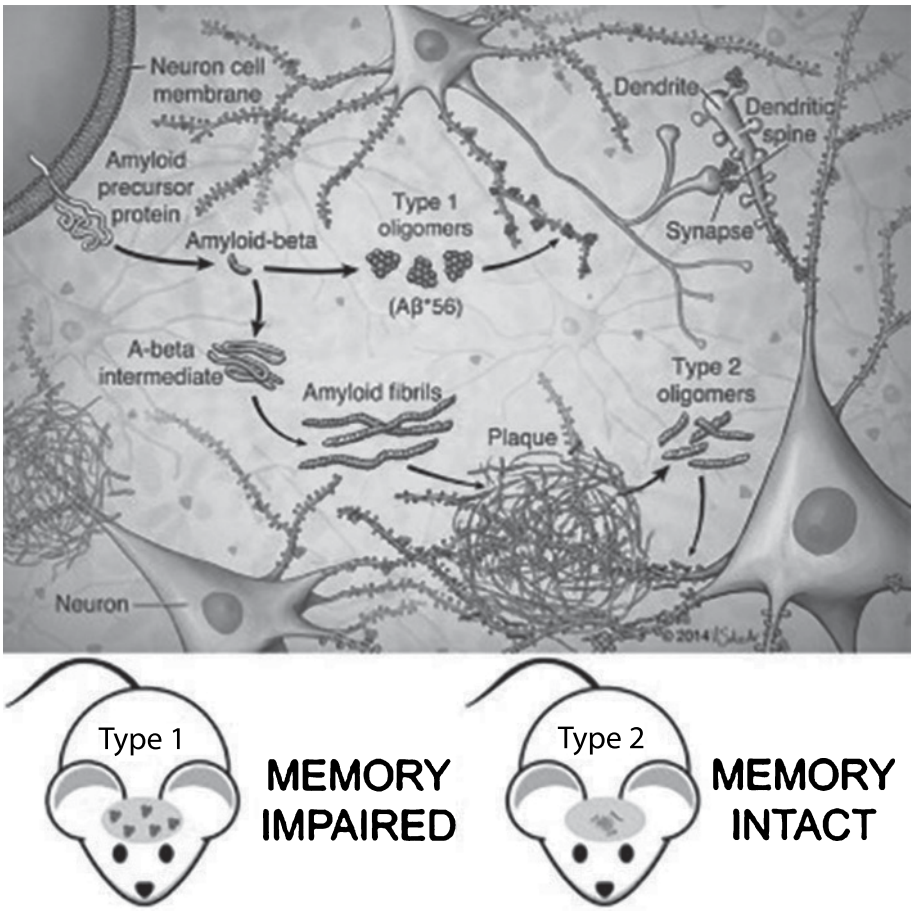

Fig. 4. AβOs can be divided into two classes based on their temporal, spatial, and structural relationships to amyloid plaques as well as their ability to cause memory dysfunction. Type 1 AβOs (aka "peak 1" or HMW) are thought to be associated with memory impairment, while type 2 AβOs (aka "peak 2" or LMW) are not. Only type 2 AβOs are associated with amyloid plaques. Reprinted from "Quaternary Structure Defines a Large Class of Amyloid-beta Oligomers Neutralized by Sequestration" by Liu P, Reed MN, Kotilinek LA, Grant MK, Forster CL, Qiang W, Shapiro SL, Reichl JH, Chiang AC, Jankowsky JL, Wilmot CM, Cleary JP, Zahs KR, and Ashe KH. This was published in *Cell Rep*, 2015, 11(11): 1760-1771, under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License (CC BY NC ND) https://creativecommons.org/licenses/by-nc-nd/4.0/ [119].

and has been observed more recently in CSF [14]. A recent study by Lesné and colleagues compared the *in vitro* toxicity of Aβ*56 to two LMW species, dimers and trimers [127]. They found that Aβ*56 interacted with N-methyl-D-aspartate receptors (NMDARs), increased NMDAR-dependent Ca^{++} influx, and increased the activation of Ca^{++}/calmodulin-dependent kinase IIα (CAMKIIα). The latter was associated with increased site-specific phosphorylation and missorting of tau. Dimers and trimers did not induce any of these effects. On the other hand, trimers were able to induce pathological conformational changes in tau, which was associated with a selective decrease in proteins governing axonal transport [128]. The lack of dimer toxicity is consistent with earlier observations from O'Malley and colleagues utilizing crosslinked dimers [129]. According to their results, they proposed that the contribution of dimers to AD is through their ability to further assemble into larger, more stable synaptotoxic assemblies. It is possible that the toxic response observed with trimers above was similarly due to their ability to assemble into large, more stable synaptotoxic assemblies [106] (i.e., HMW type 1 AβOs). This possibility cannot be discounted since the trimers were not conformationally stabilized in this study.

Many studies of AβO structure and function have been conducted with synthetic AβOs or AβOs derived from Tg mouse brain. Some researchers, however, are calling for analysis to be restricted to AD brain-derived AβOs [107, 130]. Yet, it seems as if there is structural homology between synthetic

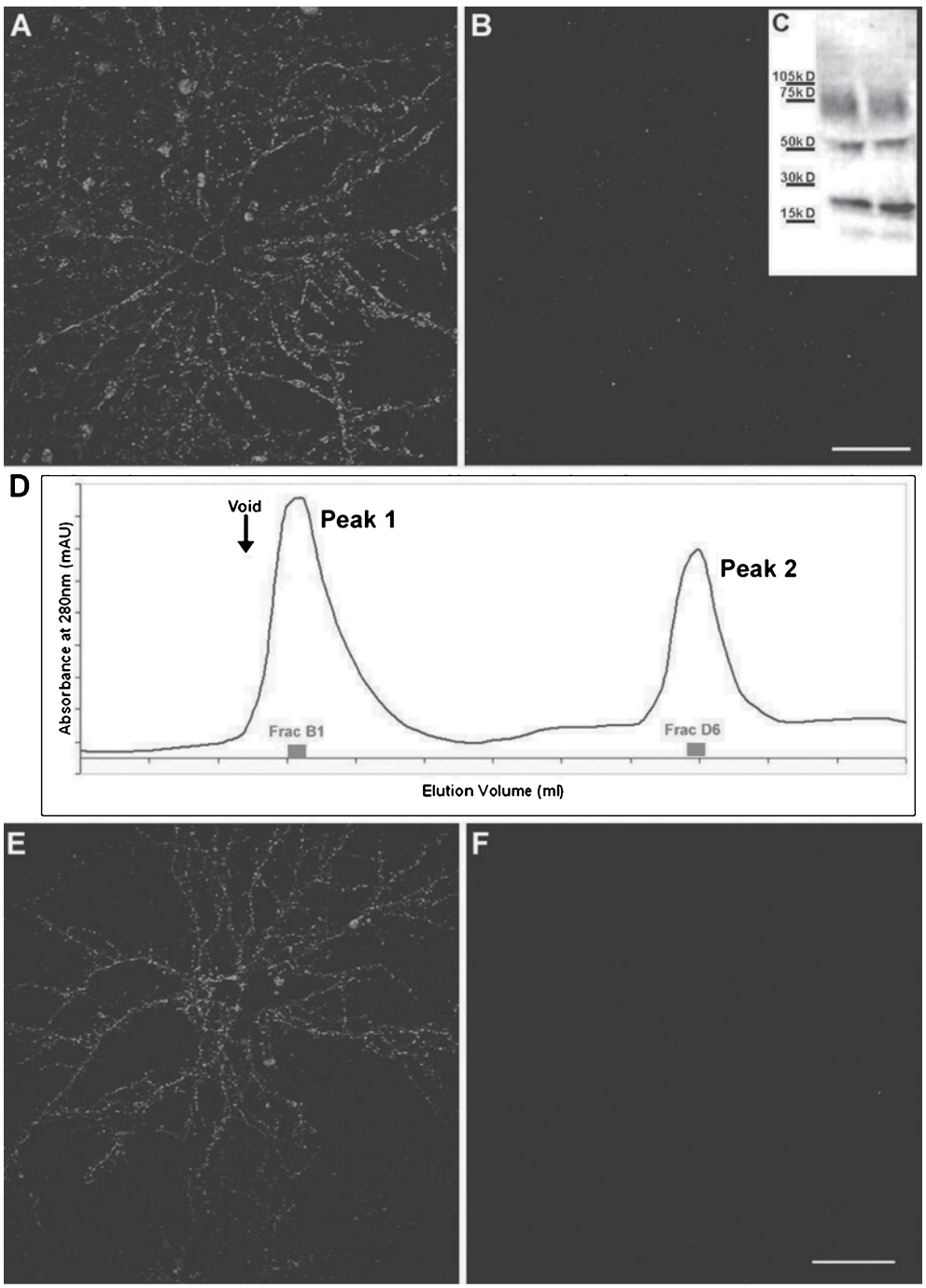

Fig. 5. Only high-molecular weight AβOs are capable of binding cultured hippocampal neurons. Synthetic AβOs were divided into high and low molecular weight populations using 50 kDa molecular weight cutoff ultrafiltration (A-B) or size exclusion chromatography (D-F) and incubated with cultured hippocampal neurons. Only high-molecular weight AβOs bind neurons (A, E); no binding of low-molecular weight AβOs was evident (B, F). Scale bar = 40 μm. Reprinted from "Synaptic targeting by Alzheimer's-related amyloid beta oligomers" by Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, Lambert MP, Velasco PT, Bigio EH, Finch CE, Krafft GA, and Klein WL. This was published in *J Neurosci*, 2004, 24(45): 10191-10200, copyright 2004; permission conveyed through Copyright Clearance Center, Inc. [16].

and brain-derived AβOs. For example, under non-denaturing conditions, synthetic and brain-derived AβOs show structural equivalence in terms of mass, isoelectric point, and immunoreactivity with conformation-sensitive antibodies [6]. Furthermore, as suggested above, at least three identical AβO species can be found in AD brain and the brain of multiple Tg mouse models: Aβ*56, dimers, and trimers [14, 44, 128].

However, one important justification for restricting analysis to AD-derived AβOs is the increasingly apparent presence of Aβ proteoforms in the AD brain as well as the contribution of Aβ proteoforms to Aβ aggregation and toxicity (reviewed in [107, 131]). These proteoforms are not present in synthetic or cell-derived AβOs. It is well known that $Aβ_{40}$ and $Aβ_{42}$ are the most abundant Aβ peptides found in AD. However, in addition to these peptides, myriad truncated Aβ peptides also have been found in the AD brain [132] and CSF [133]. Using mass spectrometry, one study identified 26 unique Aβ proteoforms in the AD brain. 73% of these were N-terminal truncations and 30% were C-terminal truncations. The N-terminally truncated peptides were predominately found in the insoluble fraction of the brain, while the C-terminally truncated were predominately found in the soluble fraction. Canonical $Aβ_{42}$ was a minority of the proteoforms identified and was equally distributed between the insoluble and soluble fractions [132].

Truncated Aβ peptides likely play a role in AD pathogenesis as they can form stable oligomeric complexes with the full-length $Aβ_{42}$ peptide [133]. In fact, N-terminally truncated Aβ peptides formed through pyroglutamylation of glutamic acid residues are increasingly recognized as very toxic proteoforms of Aβ. Pyroglutamylated (pE) Aβ has been found to drive misfolding of Aβ into more toxic aggregates when present at 5–33% of the total Aβ concentration [134]. Pyroglutamylation also increases the aggregation speed of Aβ [135]. Anti-pE Aβ antibodies have been developed and successfully utilized in Fab form for co-crystallization with pE Aβ [136]. These studies revealed that the pE modification confers a pronounced bulky hydrophobic nature to the N-terminus of Aβ that might explain its enhanced aggregation properties. Interestingly, one group finds that pE AβOs may be the most abundant AβO species in AD brain [137]. Other Aβ proteoforms reported in the past 5 years to increase Aβ toxicity include C-terminally extended $Aβ_{43}$ [138–140], Aβ peptides with N-terminal extensions up to 40 residues [141, 142], aspartic acid isomerization [143], and phosphorylation [144, 145].

AβO assembly pathways and their relation to amyloid plaques

A preponderance of data now supports the hypothesis that some AβO species are "on-pathway" to fibril formation, while others are "off-pathway". It is these "off-pathway" oligomeric species that may be the most toxic [146]. This on/off-pathway classification appears to correlate with the HMW/LMW and type 1/2 AβO classifications discussed above. Most data show that LMW, type 2 AβOs are on-pathway to fibril formation, while HMW, type 1 AβOs are off-pathway [118, 119, 147, 148]. High-speed AFM imaging demonstrates that LMW AβOs quickly form fibrils, whereas HMW do not [147]. These aggregation differences between LMW and HMW AβOs are consistent with earlier findings using SDS-PAGE analysis [118]. In fact, it seems as if the only contribution of HMW AβOs to fibril formation may be through their dissociation into LMW AβOs, which then seed fibril formation [147]. Interestingly, differences in the aggregation pathways of these two AβO structures occur as early as the dimer stage [149]. But contrary to the hypothesis of HMW AβOs being more toxic than LMW AβOs, one study has found that HMW AβOs are not as toxic as the LMW AβOs into which they dissociate [150]. And another study utilizing all-atom molecular dynamics simulations observed that compact AβO structures, with an oligomeric order up to 18 (81 kDa), are off-pathway to fibril formation, while larger, elongated AβO structures are on-pathway to fibril formation [149]. Therefore, although there is general agreement in the literature regarding toxicity of AβO species, there is not complete consensus.

An alternate hypothesis to the on/off-pathway model of AβO formation, is the fibril-seeded model [151]. In this model, toxic AβOs are predominantly formed from monomers that dissociate from fibrils only after a small, but critical concentration of fibrils has formed. This model is supported by kinetic experiments, selective radiolabeling experiments, and cell viability assays. Further support for secondary nucleation of AβOs comes from molecular dynamics simulations. These simulations predict that a hydrophobic fibril region causes the structural changes required for catalyzing the formation of AβOs on the fibril surface [152]. However, AβOs can form within minutes *in vitro*, even at low nanomo-

lar concentrations [26]. Recent AFM studies confirm that AβOs can form within minutes [153]. This quickly forming AβO population is specifically dominated by hexamers and dodecamers and quickly followed by AβO-seeded fibril formation. Therefore, one factor that has led to contrasting conclusions regarding the timing of AβO primary versus secondary nucleation pathways may be the differing time resolutions of the different experimental techniques.

Another hypothesis, the amyloid plaque buffering hypothesis, supports this notion of co-existing primary and secondary nucleation pathways for AβOs. This hypothesis predicts that plaques act as a reservoir or sink for toxic AβOs [107]. AβOs gradually deposit as diffuse plaques, which cause inflammation, but AβOs also can directly cause damage leading to dementia via altered signaling [5, 110]. Evidence for AβOs existing in these diffuse plaques comes from immunofluorescent imaging with anti-AβO antibodies. This has been observed in the AD brain and in the brains of multiple animal models [7, 96, 154, 155]. Over time, this plaque reservoir is saturated or loses capacity and toxic AβOs become free to diffuse and exert toxicity [107, 154, 156, 157]. Overall, it seems as if evidence in the literature converges into the hypothesis that Aβ aggregates into distinct AβO species, with differing toxicities and relationships to fibrils, that can interconvert.

Emerging insights into how to approach molecular structure

A major hurdle to AβO structural characterization is AβO metastability and heterogeneity. One major approach to stabilize and isolate distinct AβO species has been crosslinking. One widely applied crosslinking method for AβO stabilization has been photo-induced crosslinking (PICUP), developed by the Teplow group. Initially, this method was successful in stabilizing only LMW oligomers of the $A\beta_{40}$ peptide [158]. Recently, PICUP has been improved through use of the mutated $A\beta_{42}$ peptide [F10, Y42]$A\beta_{42}$, enabling stabilization of $A\beta_{42}$ oligomers up to dodecamers [159]. Another crosslinking strategy used for AβO stabilization is dityrosine crosslinking. This method is thought to be AD-relevant as it occurs under conditions of elevated copper and oxidative stress [160]. Copper is relevant to AD as there is some evidence that dyshomeostasis of metals, including copper, may contribute to AD pathogenesis [161]. Furthermore, dityrosine crosslinked proteins are found to be prevalent in AD brain and CSF [160]. Molecular dynamics simulations predict that dityrosine crosslinking promotes Aβ self-assembly, at least up to tetramers [162]. In one study, copper was found to stabilize Aβ in an oligomeric conformation sufficiently to enable 3D structural characterization by small-angle x-ray scattering [163]. The putative mechanism of this copper-mediated stabilization was through copper-induced dityrosine linkage of Aβ peptides [164]. Different copper ratios had different effects on Aβ aggregation, with supra-equimolar ratios favoring formation of ellipsoid oligomers and sub-equimolar ratios favoring formation of fibrils [163]. These ellipsoid AβOs were predicted to contain 38 copies of the Aβ peptide and are therefore consistent with the converging classifications of off-pathway, HMW, type 1 AβOs. Given published findings, it is essential that AD-relevant stabilization techniques continue to evolve to enable direct structure-function comparisons of distinct AβO species under AD-relevant experimental conditions. This will make it possible to properly interpret Aβ-directed clinical findings and make the most informed efforts at rational design of AβO-targeting therapeutics.

What makes AβOs toxic to neurons?

AβOs can be extracellular *in vivo*, existing in CSF [32, 34, 35, 165] and in interstitial fluid [166]. Some brain cells when exposed experimentally to extracellular AβOs become dysfunctional and deteriorate, as reviewed above. How AβOs instigate pathological changes, and why only some cells are affected, are fundamental questions to which we do not yet have satisfying answers.

The simplest possibility is that cell damage is initiated by physiochemical interactions between AβOs and neuronal membranes. It has been reported that AβOs can insert directly into lipid bilayers, causing disruption by acting as a pore, a phenomenon first observed with artificial membranes [167]. The extensive amount of literature concerning Aβ-lipid membrane interactions and molecular level membrane modeling has recently been reviewed [168], including the possible involvement of metals in the mechanism [169]. AFM was used recently to show structural damage to POPC/POPS lipid bilayers caused by $A\beta_{40}$ in different aggregation states [170]. Aggregation and lipid interaction properties of Aβ peptide fragments incubated in the absence or presence of total brain lipid extract bilayers indicate that some sequences interact with and disrupt

bilayers (e.g., $A\beta_{40}$) but others do not (e.g., $A\beta_{28}$ and $A\beta_{12\text{-}24}$) [171]. Some experiments indicate that oligomers of Aβ have more membrane affinity than monomers [172]. Putative oligomers from Aβ that is pyroglutamate-modified also binds neurons and causes a loss of plasma membrane integrity [173]. Ion channel formation in cell membranes [174] has been reported for oligomers of $A\beta_{42}$, but not $A\beta_{40}$, and attributed to a pore-forming beta-barrel AβO structure [175]. Individual AβOs larger than trimers reportedly induce Ca^{++} entry as they cross the cell membrane [176]. Cholesterol enhances formation of an annular octameric channel of $A\beta_{22\text{-}35}$, which induces a zinc-sensitive Ca^{++} influx [177], suggested as a possible lipid raft association. Recruitment of AβOs to rafts is consistent with findings that depletion of the ganglioside GM1 blocks AβO interactions toxicity [178]. On the other hand, data suggest that a moderate increase in membrane cholesterol content may be protective against AβO toxicity [179]. Protection also is conferred by a pentapeptide from the glycine zipper region of the C-terminal of Aβ, which blocks apparent membrane insertion and abolishes synaptotoxicity [180].

One significant difficulty encountered by the bilayer insertion hypothesis is its inability to account for the specificity of AβO attachment. Two neurons side-by-side can exhibit completely different ability to accumulate AβOs, one showing robust synaptic accumulation and the other showing virtually none [16]. Cell-specific toxicity, measured by tau hyperphosphorylation, correlates with this binding [51]. There also is a difficulty in accounting for binding saturability [16, 96], although it might be argued that AβO insertion into lipid rafts specific to particular synapses could be saturable. It has been hypothesized that AβOs may act through both lipids and proteins, forming pores within membranes while also binding to receptors to induce specific intracellular responses [181].

The receptor hypothesis regards AβOs as gain-of-function pathogenic ligands that bind adventitiously to specific proteins acting as toxin receptors. Overall, the receptor hypothesis provides a mechanism that fits well with salient facets of the cell-based evidence. The hypothesis was introduced to explain toxicity that was cell-specific and dependent on expression of Fyn, and to explain why AβO binding was virtually eliminated by treating cell surfaces with low doses of trypsin [26]. Consistent with the receptor hypothesis, AβO binding shows (A) saturation and high-affinity for cultured neurons and synaptosome preparations; (B) specificity for particular neurons and particular brain regions; (C) targeting of synapses; (D) accumulation at dendritic spines; (E) pathological impact, such as tau hyperphosphorylation, specific to neurons with bound AβOs; (F) sensitivity to low doses of antagonist; (G) binding to trypsin-sensitive proteins; (H) association with small patches of isolatable membranes; and (I) specificity in Far-Western immunoblots [6, 16, 48, 167, 182]. These findings apply generally to brain-derived and synthetic AβOs and support the hypothesis that binding of AβOs is ligand-like and mediated adventitiously by proteins acting as toxin receptors.

Perhaps the most intriguing and well-studied AβO toxin receptor candidate is the cellular prion protein (PrPc). Strittmatter and colleagues in a series of papers have provided strong evidence that PrPc is capable of mediating AβO binding [122, 183–185], starting with their unbiased screening of a cDNA expression library that identified PrPc as a potential high-affinity AβO receptor [186]. Extensive experiments with multiple models support this possibility and connect binding to neural damage [187–190]. It has been reported that binding of AβOs to PrPc is dependent on integrity of cholesterol-rich lipid rafts and that AβOs bound to PrPc accumulate in endosomes after which they are trafficked to lysosomes [191]. Investigations of how externally-oriented PrPc might bring about intracellular damage through transmembrane coupling to Fyn are discussed further below. Coupling of AβO-bound PrPc to Fyn is consistent with the original studies showing that Fyn expression is required for AβO-induced toxicity [26, 192] and evidence showing involvement of Fyn in physiological PrPc signaling [193]. It should be noted that the PrPc hypothesis is still somewhat controversial, and some reports are not in agreement with the role of PrPc as an AβO toxin receptor [29, 194–197].

Another promising candidate receptor is the Na^+K^+ ATPase alpha3 subunit (NKAα3), recently identified independently by two groups using disparate preparations and identification strategies. It was shown first by Ohnishi and colleagues that NKAα3 can bind both brain-derived and synthetic AβOs [198], each resembling type 1 AβOs with respect to their relatively large size. Verification of NKAα3 as an AβO receptor subsequently was provided by DiChiara et al. [199]. This group used solubilized synaptic membrane proteins reconstituted in nanoscale artificial membranes and an AβO-specific antibody to isolate AβO-bound NKAα3. Co-localization of AβO binding sites with NKAα3

was confirmed in hippocampal cell cultures. As discussed later, down-regulation of NKAα3 could play a significant role in converting AβO binding into cell pathology.

Overall, and rather remarkably, the current list of candidate toxin receptors for AβOs comprises a very large number of membrane proteins besides PrPc and NKAα3. As has been reviewed [115, 200–202], these include the metabotropic glutamate receptor 5 (mGluR5) [182, 184], NMDARs [58, 62], Sigma-2 receptor/progesterone receptor membrane component 1 (PGRMC1) [203, 204], frizzled receptor [205], neuroligin [206], Ephrin type-B receptor 2 (EphB2) [207], Ephrin type-A receptor A (EphA4) [208, 209], p75 neurotrophin receptor (p75NTR) [210], alpha7-nicotinic acetylcholine receptor (α7nAChR) [211, 212], adrenergic receptors [213], the receptor for advanced glycation endproducts (RAGE) [214], calcium channels [215–217], leukocyte immunoglobulin-like receptor subfamily B member 2 (LILRB2)/paired Ig-like receptor B (PirB) [64, 218, 219], N-formyl peptide receptor 2 (FPR2) [220], immunoglobulin gamma Fc region receptor II-b (FcγRIIb) [221], transient receptor potential melastatin 2 (TRPM2) [222], insulin receptor (IR) [48], and the AMPA receptor [223].

It is not known why there are so many candidate receptors. There certainly are different forms of AβOs, which could interact with different membrane proteins. AβO ligands in aqueous buffer are between 100 and 300 kDa (Cline and Klein, unpublished); in this mass range AβOs would comprise 22–66 Aβ monomers. To interact with a binding domain of a toxin receptor, only particular regions of the oligomer surface would be needed. Ligand-like regions could assume multiple configurations influenced by buffer composition. For example, monomeric Aβ in Ham's F12 media assembles into structures quite different than Aβ in phosphate buffered saline (the former has a high type 1 to type 2 ratio [118], while the latter has a low type 1 to type 2 ratio) (unpublished data). Even within a population of synthetic type 1 AβOs, there is a small subpopulation of synapse-binding AβOs that can be targeted uniquely by a selective single-chain variable fragment antibody [118]. Different targeted binding proteins might nonetheless mediate similar changes downstream. It is known, for instance, that AD-type phosphorylated tau can be induced by oligomers of different proteins such as Aβ [51], α-synuclein [224], and even lysozyme [225].

Receptor transduction mechanisms → how does the initial receptor-AβO interaction on neurons trigger a change that leads to intracellular damage?

The mechanism by which PrPc mediates AβO impact intracellularly has been carefully worked through (Fig. 6). It incorporates a number of molecular players previously implicated by multiple laboratories: mGluR5 [182, 184], Fyn [26], tau [51], NDMARs [58, 62] and protein tyrosine kinase 2 (Pyk2) [226]. Both high and low molecular weight AβOs have been implicated in this pathway [121–123, 191]. Data are consistent with a mechanism in which AβOs first bind to PrPc on cell surfaces and stimulate Fyn via mGluR5 activation (reviewed by Nygaard, et al. [227]). Consistent with activation of mGluR5 by AβOs, the ability of glutamate to activate the prion-mGluR5 complex is occluded [228]. Downstream, stimulated Fyn is known to phosphorylate tau [229] and cause tyrosine phosphorylation of the NR2B subunit of NMDARs [183]. It is thought that AβO binding to neurons and AβO neurotoxicity depends on a pre-existing PrPc-mGluR5 complex [230]. However, since PrPc can be removed with full retention of AβO binding [194], it may be that the critical membrane-organizing function of PrPc precedes the ligand binding step.

An interesting potential connection exists between this synaptopathic mechanism and Pyk2. Pyk2 has a single nucleotide polymorphism identified as increasing the likelihood of late-onset AD [231]. Functionally, Pyk2 is a focal adhesion kinase (FAK), an enzyme that previously was shown to be stimulated by toxic Aβ preparations and to form complexes with Fyn [226]. Pyk2 normally helps regulate synaptic plasticity [232–235]. It is activated by increased intracellular calcium and by Fyn [236–241]. Ectopic activation of Pyk2 potentially could be an early event in this AβO pathogenic pathway, which would provide a molecular basis for its risk factor status.

Insights also have been obtained into how AβO binding mediated by NKAα3 could be transduced into neuronal damage. As described by Ohnishi et al. [198], AβO binding leads to a slow, time-dependent decrease in ATPase activity. The consequence is Ca^{++} buildup via N-type voltage-sensitive calcium channels (N-VSCC) and mitochondrial channels and ultimately apoptosis. Decrease in activity was suggested as linked to AβO binding to the ouabain binding site of the NKAα3 [198]. This observa-

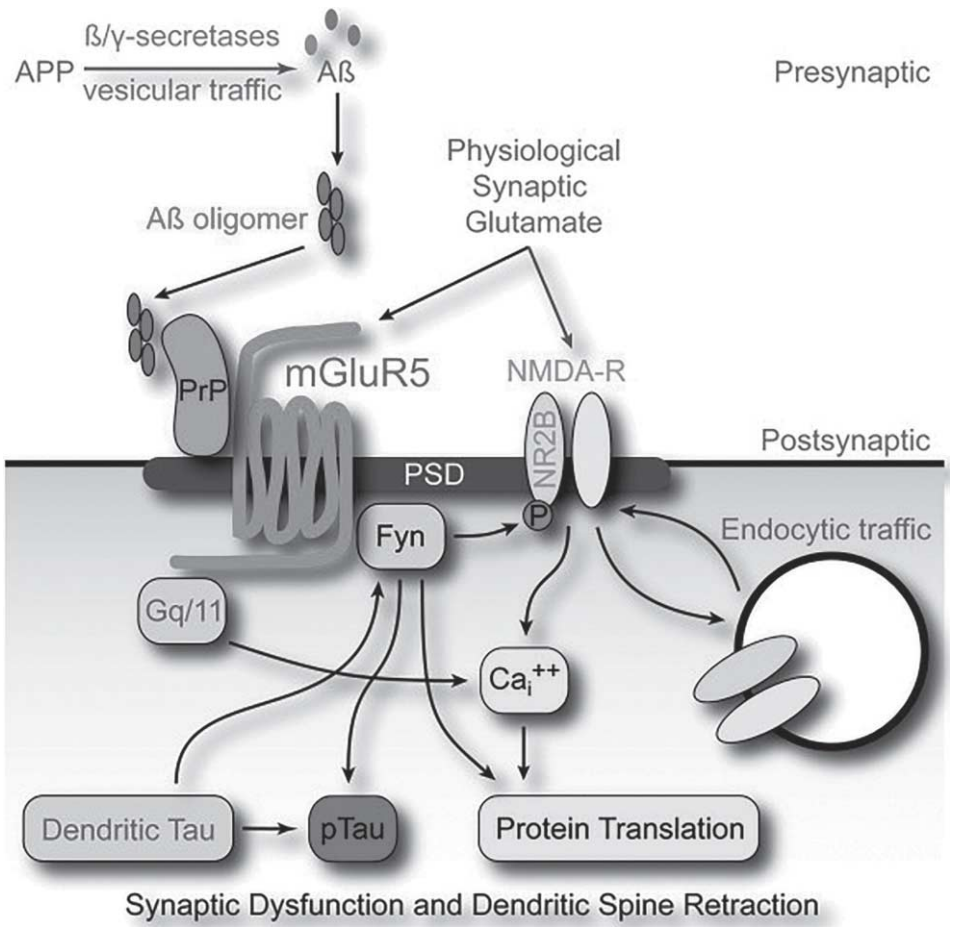

Fig. 6. PrPc mediates AβO toxicity through mGluR5, Fyn kinase, and NMDARs. Downstream consequences of the pathway include calcium dyshomeostasis, tau hyperphosphorylation, and synaptic dysfunction and loss. Reprinted from "Fyn kinase inhibition as a novel therapy for Alzheimer's disease" by Nygaard HB, van Dyck CH, and Strittmater SM. This was published in *Alzheimers Res Ther*, 2014, 6(1): 8, under the terms of the Creative Commons Attribution License (CC BY) [227].

tion suggests the new hypothesis that, under some circumstances, AβOs could be endogenous ouabain-like physiological regulators of ATPase. The slow, time-dependent decrease in activity, however, could be linked to the observed impact of AβOs on NKAα3 distribution. Following exposure to AβOs, neuronal NKAα3 accumulates in dense clusters along dendrites. These clusters of NKAα3 increase in size and then decrease in abundance (Fig. 7) [199]. This presumably occurs at dendritic spines, where AβOs also cluster [16, 55]. Like the NKAα3 redistribution, spines undergo time-dependent changes in morphology and abundance due to AβO exposure. Ultimately, there is a large down-regulation of NKAα3, which could account for decreased ATPase activity (Fig. 7).

The issue of distribution is a salient one given that NKAα3 acts not only in cation transport, but also as a membrane protein docking station that functions to control signaling pathways [242]. These docking stations organize multiple membrane proteins [8], including neurotransmitter receptors linked to AβO-induced neuron damage [243]. The clustering of NKAα3 is in harmony with the earlier observation that AβOs induce the clustering of mGluR5 [182, 184]. As discussed above, mGluR5 is a Ca^{++} mobilizing receptor, and it is regarded as a key mediator of AβO-elevated Ca^{++} buildup and the damage that ensues [184]. The time-dependence of AβO-induced clustering of mGluR5 has been imaged using quantum dots and single-particle tracking in experiments with live neurons [182]. It has been hypothesized

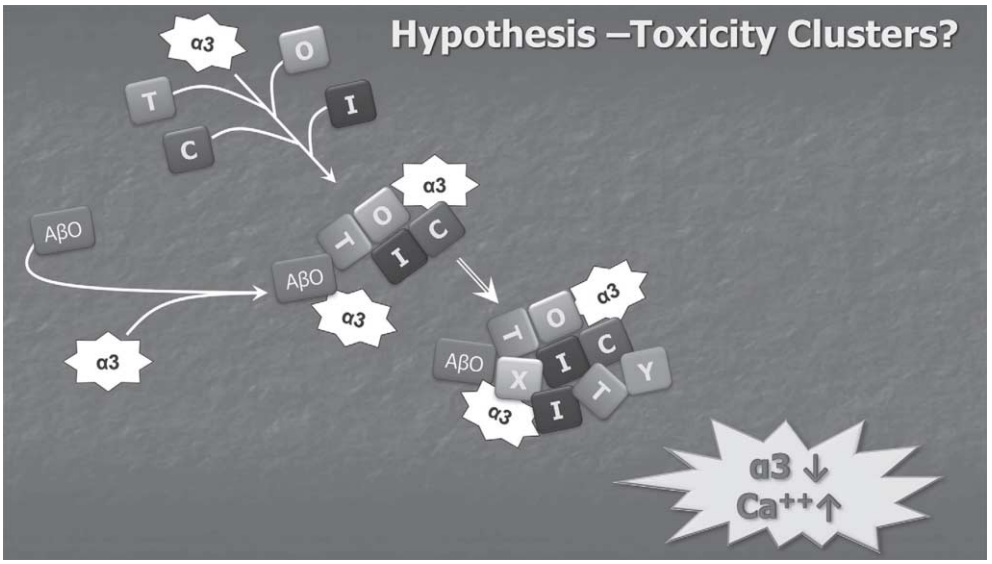

Fig. 7. AβOs induce membrane re-distribution of NKAα3 subunit resulting its downregulation and excessive Ca^{++} buildup. A hypothesized early event in AβO-induced neuronal damage is binding to NKAα3 on neuronal membranes, causing restructuring of the NKAα3 docking station into toxic clusters of membrane proteins. Ultimately, this results in downregulation of NKAα3 on the neuronal surface and buildup of toxic Ca++. Adapted and reprinted from "Alzheimer's Toxic Amyloid Beta Oligomers: Unwelcome Visitors to the Na/K ATPase alpha3 Docking Station" by DiChiara T, DiNunno N, Clark J, Bu RL, Cline EN, Rollins MG, Gong Y, Brody DL, Sligar SG, Velasco PT, Viola KL, and Klein WL. This was published in *Yale J Biol Med*, 2017, 90(1): 45-61, under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License (CC BY NC ND) https://creativecommons.org/licenses/by-nc-nd/4.0/ [199].

that mGluR5 clustering itself is a seminal step for the transduction mechanism [182]. Supporting this possibility, clustering of mGluR5 molecules induced by receptor antibodies mimics the toxic impact of AβOs [182]. Because mGluR5 and NKAα3 each co-localize with cell-surface bound AβOs, they likely are part of the same ectopic clusters. Recently, single-particle tracking experiments have shown that NKAα3 becomes immobilized during exposure of hippocampal neurons to toxic assemblies of synuclein [244]. These results support the hypothesis that NKAα3 has a central role as an immobilizing docking station for toxic oligomers found in multiple proteinopathies. With respect to generation of these clusters, the role of the NKAα3 docking station relative to roles played by mGluR5, or other membrane domain-organizing proteins such as PrPc [245], is not yet clear.

The seminal interactions between AβOs and NKAα3 molecules at the cell surface may be suitable targets for new drug discovery strategies, as suggested by Ohnishi et al. [198]. Attachment of AβOs to NKAα3 is amenable to high-throughput screening for antagonists using Nanodiscs [194, 246]. Results from a preliminary screen showed that AβO binding to spines can be blocked by low doses of a small organic molecule, albeit one with promiscuous binding, precluding its use for therapeutics [194]. Nonetheless, Lee and colleagues have shown that behavior in a Tg AD model could be safely rescued using this same compound [247]. Future investigations of the docking station hypothesis are expected to open the door to therapeutics targeting the first step of a complex pathway that leads to neural damage and dementia.

Whether the NKAα3 acts, as has been suggested [198], as a "death target" for AβOs is not confirmed yet. Most AD-like pathology is evident in cultures containing almost exclusively neurons, but cell death is minimal; neuron death likely requires the presence of factors released by glia [248]. It is possible that the impact of AβOs on NKAα3 may render them more vulnerable to inflammatory cytokines.

TRENDING TOPICS IN AβO RESEARCH

With about 2,000 AβO papers published since 2013, there has been a great deal of progress on many

issues. Some of the salient directions are considered briefly in this section.

Toxic effector pathways after initial transduction

Downstream, after the initial transduction steps, the impact of AβOs has been tracked to mitochondrial effects, ER stress, and autophagy/lysosomal dysfunction. These may be the consequences of surface events discussed above, but some studies show that AβOs may themselves enter cells and act directly on these organelles, as discussed below.

AβO-associated NMDAR activation [62] promotes Ca^{++} release from the ER, which leads to ER stress [249] with subsequent mitochondrial dysfunction [250], astrogliosis [115, 251], and apoptosis [18]. AβOs also have been found to trigger the unfolded protein response, a collection of signaling pathways that respond to ER stress [252]. AβOs further decrease resistance of brain mitochondria to Ca^{++}-induced opening of mitochondrial permeability transition pores [253]. Cytochrome C is released by AβO-activated BAK pores [254]. Voltage-dependent anion channel 1 also interacts with Aβ monomers and oligomers, and the block of mitochondrial pores leads to mitochondrial dysfunction [255]. Morphological effects on mitochondrial fusion and fission dynamics, essential for neuronal function, have been reviewed [256]. AβO targeting of mitochondria promotes mitochondrial fission, disruption of mitochondrial membrane potential, increase of intracellular ROS and activation of mitophagy [257]. AβOs decrease mitochondrial volume [258], and AβO-induced oxidative stress is associated with mitochondrial fission [259]. AβOs activate fragmentation through the GTPase dynamin-related protein 1 (Drp-1) [260] and extracellular signal-regulated kinase (ERK) [259]. Fragmentation also has been associated with AβO-induced mitochondrial transport defects, with histone deacetylase (HDAC6) activation part of the mechanism [260]. AβO-induced mitochondrial damage appears to be restricted to neurons and not microglia or astrocytes [261].

With respect to autophagy, the sole catabolic mechanism for degrading protein aggregates, there is increasing evidence for autophagic dysfunction in AD and other neurodegenerative diseases [262, 263]. The endosomal-lysosomal (autophagy) system is a prominent site of AβPP processing, Aβ uptake, and Aβ production [262]. One study has found that AβOs associate with autophagic vacuoles in AD axons, starting a pathway that impairs retrograde transport, which contributes to autophagic stress [264]. On the other hand, another study found that it is Aβ monomers, and not AβOs, that contribute to autophagy [265]. AD and lysosomal storage disorders share many overlapping pathologies, including neuronal accumulation of lysosomal vesicles, dystrophic axons, ectopic dendrites, cognitive deficits, and neurodegeneration [262]. Lysosomal storage disorder gene variants also have been found to be associated significantly with Parkinson's disease [266]. Restoration of autophagy function may represent a promising therapeutic target as rifampamycin, a candidate preventative therapeutic thought to restore autophagy function, has been found to inhibit oligomerization of Aβ and tau, tau phosphorylation, synapse loss, and microglial activation in AD mouse models [267].

Intracellular effects of AβOs may be instigated by surface mechanisms but also could be a result of direct interactions between organelles and internalized AβOs. In NHPs, i.c.v.-injected AβOs were observed on the surface and inside neurons [46]. Internalization may involve signaling pathways that affect regulation of receptor-mediated endocytosis. In the human neuroblastoma SH-SY5Y line, AβOs activate p38 mitogen-activated protein kinase (p38 MAPK) and ERK1/2 signaling pathways via the α7nAChR, which in turn results in AβO internalization [268]. Internalized Aβ (monomers and AβOs) localized to all organelles (ER, Golgi complexes, multivesicular bodies/late endosomes, lysosomes, exocytotic vesicles, and mitochondria) and non-membrane-bound cytosolic structures [269, 270]. The uptake of Aβ via endocytosis is rapid and spontaneous. It is retained in lysosomes, where accumulation leads to aggregation [271].

The relationship between neurons, astrocytes, microglia, and AβOs

De Strooper and Karran propose that AD pathogenesis is not simply a neuron-centric, linear cascade initiated by Aβ and leading to dementia, but rather a long, complex cellular phase consisting of feedback and feedforward responses of astrocytes, microglia, and vasculature [202].

Many lines of evidence support this hypothesis. For instance, AβOs have been found to induce

astrogliosis [272] and trigger ROS generation in activated astrocytes [273]. AβOs reportedly cause disturbances in the signaling of insulin, protein kinase B (Akt), and excitatory amino acid transporters 1 and 2 [274]. Decreases in the activation and expression of astrocytic glutamate transporters has been linked to impaired synaptic plasticity [275]. AβOs at picomolar levels, within minutes, can increase levels of intracellular Ca^{++} in astrocytes but not neurons [276]. An increase in ROS production by nicotidamine adenine dinucleotide phosphate (NADPH) oxidase in both neurons and astrocytes has been found to activate caspase-3, also linked to LTP inhibition. In these experiments, only a small fraction of AβOs were impactful and their damage was blocked by clusterin [276]. AβOs and fibrils bind and activate Ca^{++}-sensing receptors, which drives both neurons and astrocytes to secrete $A\beta_{42}$. While the Aβ-exposed neurons start dying, astrocytes survive, and they keep over-secreting $A\beta_{42}$, nitric oxide (NO), and vascular endothelial growth factor A (VEGF-A), apparently contributing to the demise of neurons [277]. On the other hand, astrocytes, before they are affected by AβOs, appear to release insulin and insulin-like growth factor (IGF1), whose trophic effects serve to protect neurons from AβO toxicity [73].

Microglia in AD are involved in phagocytosis of Aβ plaques [278–281], a process that is regulated by astrocytes [278]. It is possible that AβOs play a role in attracting microglia to plaques (Bicca and Klein, unpublished data). Besides chemotactic effects, AβOs induce a switch in microglial phenotype to a pro-inflammatory phenotype, leading, e.g., to aberrant tumor necrosis factor (TNF) signaling [282]. Aberrant TNF signaling causes decreased brain serotonin levels and subsequent depression [283]. It also causes insulin receptor substrate (IRS-1) and PKR (dsRNA-dependent protein kinase)-dependent synaptic dysfunction and memory loss [221]. There thus is a link between AβOs, neuroinflammation, mood alterations, metabolic disorders, and memory loss. Microglia also may contribute to AβO formation, by releasing particles that can bind rapidly to Aβ and cross-seed its aggregation, including oligomerization [284]. It should be noted that there also is evidence that microglia may contribute to neuronal loss and memory impairment in a manner independent of Aβ [281]. One potential mechanism is through microglia engulfment of synapses [285].

Tau progression and prion-like action may be instigated and potentiated by AβOs: PART (primary age-related tauopathy)

Most evidence in the literature converges on the hypothesis that AβOs are upstream of tau in AD pathogenesis and not the other way around, as reviewed by Bloom [286]. However, there is currently no consensus in the field, with some studies demonstrating crosstalk between AβOs and tau and some demonstrating that each acts separately [110, 286–290]. In support of the hypothesis that AβOs trigger tau pathology, it was demonstrated in 2008 that AβOs were capable of inducing tau hyperphosphorylation in cultured neurons in the absence of fibrils [51]. Tau distribution in AβO-exposed neurons ectopically redistributes to dendrites [52]. AβOs also have been shown to induce tau-dependent microtubule severing [291], to disrupt tau translocation to excitatory synapses [292], and to stabilize microtubules, the latter leading to tau-dependent loss of spines and tau hyperphosphorylation [293]. AβOs even can seed the formation of tau oligomers, which are thought to be the most toxic form of tau [294]. In the AD brain, synaptic AβOs have been found to precede synaptic phosphorylated tau (pTau), even perhaps driving the synaptic spread of pTau [295]. It is known that spread of tau pathology in a Tg mouse tauopathy model is accelerated by crossing with an APP Tg mouse [296]. Recent data suggests that AβOs may induce neurons to release pTau within exosomes, thereby suggesting a potential mechanism for AβO-induced spread of tau pathology [73]. Interestingly, this release of tau was increased by the presence of insulin. The idea that tau is secreted by neurons is supported by numerous other studies, as reviewed by Pooler et al. [297]. Furthermore, i.c.v. injection of AβOs into NHPs induces tau hyperphosphorylation and formation of neurofibrillary tangles throughout the NHP brain [46].

Many recent studies have attempted to give Aβ and tau an even playing field on which to determine their pathological relationships by crossbreeding mice expressing human tau (wild-type or mutant) and APP/PS1 mutants. However, these studies show inconsistencies in data leading to contrasting conclusions. Co-expression of mutant tau and mutant Aβ appears to support a synergistic action, showing dramatically increased pTau aggregation and spread, inflammation, and synapse loss [296, 298, 299]. Multiple studies utilizing a wild-type human tau instead of

mutant tau also support a synergistic model, for example [300, 301]. Although it may be that some aspects of AD pathology are cooperatively affected by Aβ and tau, while others are independently affected [155, 302]. Contrary to these studies, one report found no evidence for pathological interaction between Aβ and tau [303]. These apparently disparate findings may be the result of utilizing different transgenes and/or pathological readouts.

AβOs themselves as prions?

The idea that oligomers of amyloid proteins, including AβOs, may spread from neuron-to-neuron in a prion-like manner has been widely considered. Although there is currently no clear clinical evidence that AD is a transmissible prion-like disease [5], experimental data support the idea that AβOs may spread from cell-to-cell and brain region-to-region in a prion-like manner. A recent review of this hypothesis states that Aβ aggregates have all of the key characteristics of canonical mammalian prions, including a β-sheet rich architecture, the tendency to polymerize into amyloid, templated corruption of like protein molecules, the ability to form structurally and functionally variant strains, the systematic spread by neuronal transport, and resistance to inactivation by heat and formaldehyde [304]. Another review of this concept predicts that small, extracellular oligomers of amyloid proteins would have a high propensity for prion-like spread, while large intracellular oligomers would have a lower propensity for prion-like spread [305]. In support of AβOs acting as prions, one study has found that AβOs can transfer from cell to cell [306]. This transfer was shown to be dependent on insufficient cellular clearance of Aβ peptides and oligomers. The remaining un-degraded Aβ was able to cause seeding and pathology in the receiving cells. Cell transfer was an early event seemingly independent of later toxicity. Aβ can seed its own aggregation *in vitro* [307, 308] and brain extracts from AD patients and animal models can seed Aβ aggregation *in vivo* [309, 310]. Furthermore, i.c.v. injections of AβOs to NHPs induced accumulation of AβOs in specific brain regions far from the injection site, suggesting spreading [46]. Hypotheses for the mechanisms of Aβ spread include exosome transfer [311] and spread directed by the limbic connectome [312, 313].

Mechanisms of buildup

Three intriguing new hypotheses for the mechanism of AβO accumulation that have emerged in the literature in the past 5 years are saturated proteostasis, shear-induced amyloid formation, and slowed clearance of AβOs from interstitial fluid. These hypotheses are briefly reviewed below.

Saturated proteostasis

One theory to explain the accumulation of Aβ aggregates is saturated proteostasis [314]. This theory, based on a large body of evidence, states that there may be nothing particularly unique about the $A\beta_{42}$ peptide that causes it to aggregate into toxic oligomers and amyloid fibrils. In fact, this may be an ability inherent in all proteins if they are placed in the right conditions. There is increasing evidence that many proteins are kinetically, but not thermodynamically, stable in their native states and that they become metastable when their cellular concentrations exceed their critical values. Considering the specific example of $A\beta_{42}$, one study found that changing the propensity of this peptide to aggregate by only 15% through site-directed mutagenesis resulted in large changes in toxicity [315]. The authors interpret this finding to mean that $A\beta_{42}$, and other amyloid proteins, may be extremely close to their solubility limit under physiological conditions. Thus, they hypothesize that in AD, or other neurodegenerative diseases associated with misfolded proteins and aging, age-related stress makes the entire proteome susceptible to aggregation, which in turn saturates the protein quality-control system of the cell [314]. Indeed, the majority of proteins implicated in AD were found to be present at supersaturated concentrations in the cell [316]. Therefore, proponents of this saturated proteostasis theory suggest that more effective therapeutics may target the driving forces for whole-proteome aggregation and protein quality-control mechanisms instead of individual disease-related proteins like Aβ [314]. More specifically, the systems that were found to be of importance in maintaining proteostasis in AD were involved in trafficking and clearance mechanisms, including specific branches of the endosomal-lysosomal and ubiquitin-proteasome systems [317].

Shear-induced amyloid formation

Another new hypothesis to explain the etiology of AβO buildup is the shear-induced amyloid formation hypothesis [318]. This hypothesis predicts that Aβ within the slow-flowing interstitial fluid can gain significant shear energy at, or near, the wall of the narrow extracellular spaces of the brain parenchyma. This could cause Aβ to absorb to the brain mem-

brane and form oligomers on the membrane and/or form plaques within the flow pathways of the brain extracellular spaces.

Slowed clearance of AβOs from interstitial fluid

Microdialysis experiments have shown the presence of AβOs in interstitial fluid [166]. These findings are an extension of earlier studies showing a circadian rhythm in interstitial Aβ levels [319]. Clearance through the glymphatic system is inversely correlated with AβO size [166]. Impaired glymphatic functioning is considered to be a likely factor in AβO accumulation [320, 321] (see discussion below).

Etiological factors that trigger AβO buildup in sporadic AD

There is evidence that traumatic brain injury (TBI), atmospheric pollutants, poor quality of diet and sleep, and metabolic diseases (e.g., type 2 diabetes and hypercholesterolemia), may trigger AβO buildup, eventually leading to non-inherited forms of AD (sporadic) (see, e.g., [322]). A hypothesis from De Felice for the contributions of these etiological factors to AβO buildup and AD is illustrated (Fig. 8) [322]. Evidence implicating each of these factors in AβO buildup is briefly reviewed below.

TBI

TBI is a risk factor for AD [323] with AD developing in 55.5% of TBI patients [324]. Aβ pathology has been found to accumulate in the brain and CSF following TBI, including amyloid plaques [325] and AβOs [199, 323, 326–328]. Soluble Aβ levels, including AβOs, increase with TBI severity [327] and declining patient prognosis [328]. Results are consistent with indications that AβPP expression is injury-related, e.g., in shaken-baby syndrome [329–331]. These observations are supported in TBI animal models, wherein Aβ levels rise within 1 hour after a single mild cortical impact and continue to rise for at least 24 hours [332, 333] and are associated with increased memory impairment [334]. AβO-associated proteins, PrPc and pTau, also are increased in TBI mouse models [335].

Atmospheric pollutants

Recent studies in mice have demonstrated that air pollutants, specifically vehicular-derived airborne nano-sized particulate matter, induce AD-like neuronal damage, including reduced synaptic function [336], altered neuronal differentiation and depression-like responses [337], and reduced neurite outgrowth [338]. Two AD risk factors, age [339] and gender (female) [337], appear to increase susceptibility to these detrimental effects.

Poor quality of diet and sleep

Diets high in sugar, salt, and fat and low in fruits and vegetables are associated with a higher risk of AD [340]. In animal models, diets high in fat increase soluble Aβ without changing plaque burden [341] and diet-induced insulin resistance impairs cognition [342]. In humans, such diets have been shown to perturb the circadian modulation of cortisol secretion, which is associated with poor sleep quality. Poor sleep quality also is associated with dementia and can negatively affect glymphatic system activity, which leads to Aβ accumulation via impaired clearance (see discussion below) [340]. Furthermore, sleep restriction in mice promotes neuroinflammation and synapse loss and potentiates AβO-induced memory deficits [343].

Diabetes

Sporadic AD has been called type 3 diabetes for its molecular and biochemical similarities with type 1 and 2 diabetes [322, 344]. An increasing body of evidence shows that AD is coupled to impaired brain insulin signaling, glucose utilization, and energy metabolism, all of which lead to increased oxidative stress, neuroinflammation, and further increased insulin resistance. Specifically considering AβO buildup, it has been found that glucose concentrations observed in diabetics facilitate Aβ oligomerization [345]. Furthermore, induction of diabetes in rabbits leads to AβO accumulation in the brain and retina [346]. Most recently, type 2 diabetes has been found to be positively associated with $A\beta_{42}$ in CSF [347]. The mechanism for AβO buildup in diabetes is not known, but it has been hypothesized to be mediated by inflammation [322].

Hypercholesterolemia

Hypercholesterolemia also is an AD risk factor [348, 349]. Many studies have shown that elevated cholesterol levels may contribute to AD pathogenesis, and several cholesterol-related gene polymorphisms are associated with AD, the most well-known of which is APOE [349]. Hypercholesterolemia accelerates AβO accumulation and memory impairment in AD mouse models [350, 351].

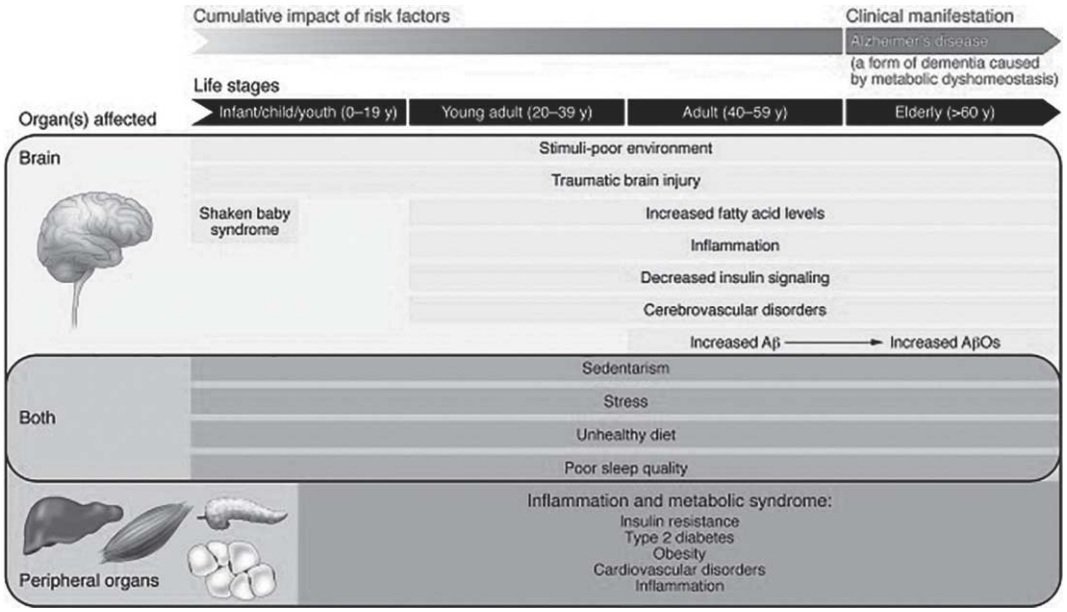

Fig. 8. A cumulative hypothesis for the development of sporadic AD. From De Felice, sporadic AD is hypothesized to be the result of the cumulative impact over a life-time of injuries to the brain and peripheral organs that results in increased AβO levels. Reprinted from "Alzheimer's disease and insulin resistance: translating basic science into clinical applications" by De Felice FG. This was published in *J Clin Invest*, 2013, 123(2): 531-539, under Free access [322].

It is important to note that even though these factors have been shown experimentally to trigger AβO buildup in sporadic AD, lifestyle and therapeutic interventions aimed at modifying these risk factors in humans have yet to show definitive success. This further highlights the difficulties and challenges associated with developing successful interventions for AD, even when the therapeutic target (AβOs) plays an established role in the disease process.

Endogenous protection and its failure

Astrocyte-mediated clearance of Aβ

A growing body of evidence indicates a role for astrocytes in clearing excess levels of Aβ from the brain [352]. Interestingly, it seems as if astrocytes have differing abilities to clear different aggregation states of Aβ, presumably due to size differences. Not surprisingly, astrocytes seem to have a harder time clearing fibrils compared to AβOs [353]. Astrocyte-mediated clearance of Aβ seems to occur by multiple mechanisms, as recently reviewed, including receptor-mediated uptake, secretion of degrading enzymes, and secretion of ApoE, which acts as a chaperone [352]. A few astrocyte receptors implicated in Aβ clearance are of note: RAGE, which is currently being targeted therapeutically in phase III clinical trials (http://clinicaltrials.gov), and matrix metalloproteinases, which are implicated in AβO-induced disruption of the blood-CSF barrier integrity (see discussion below). Overall, one possibility is that astrocyte protection of the brain from Aβ fails when Aβ accumulation reaches a certain threshold at which astrocyte-mediated clearance is saturated [352]. This hypothesis is consistent with advanced astrocyte pathology in AD brain that is detected by a monoclonal antibody developed against AβOs [89].

It may be the case that astrocytes near amyloid plaques switch from a neuro-supportive role to an inflammatory role. An opposing view is that astrocyte failure in AD is neuroprotective [354]. Another hypothesis is that Aβ, specifically AβOs, can stimulate astrocytes to produce and secrete more Aβ [355, 356]; this mechanism seems to occur through Ca^{++}-sensing receptors expressed on the astrocytes [277]. Other evidence suggests that astrocytic metabolic dysfunction may regulate Aβ production through AβPP processing [354]. And astrocytes may also mediate Aβ clearance through induction of microglial phagocytosis [278]. Thus, more experimentation is needed to fully elucidate the role of astrocytes in Aβ production and clearance within AD. It also was shown recently that healthy astrocytes

secrete insulin and IGF1 that act to protect neurons from AβO toxicity [73]. Note that these mechanisms need not be mutually exclusive.

Insulin

Extensive evidence indicates that insulin signaling and AβOs are connected in a vicious cycle of pathogenesis, as recently reviewed [200, 357]. This vicious cycle may be initiated, in some cases, by diabetes, which decreases insulin signaling in the brain. Since insulin signaling protects against AβO accumulation [346] and neurotoxicity [66], this leads to increased AβO accumulation and AβO-associated damage in the brain. AβOs themselves then further disrupt insulin signaling at many levels via pro-inflammatory mechanisms [357], e.g., by downregulating the expression of IRs on the plasma membrane [66]. Thus, a vicious pathogenic cycle is created in which AβOs upregulate their own production and aggregation by disrupting the physiological actions of insulin. Such a mechanism could account in part for AβO buildup in AD brains.

Importantly, the cellular stress and synaptic dysfunction induced by AβOs can be counteracted by stimulating brain insulin signaling [66, 322]. Therefore, either insulin or therapeutics aimed at increasing/repairing insulin signaling may be promising candidates for the treatment of AD [322]. One study exemplifying this promise demonstrated that the anti-diabetes agent exenatide protects against AβO-induced pathologies in cell culture and AβO-induced impaired insulin signaling and cognitive deficits in mice [358]. Furthermore, a recent study testing the effect in Tg mice of a therapeutic targeting multiple receptors involved in insulin signaling found a multitude of benefits including reversal of memory deficits, reduction of apoptotic factors, increase of factors promoting synaptic health, increase in neurogenesis, and reduction in Aβ, neuroinflammation, and oxidative stress [359]. Hopefully the multiple drugs targeting insulin signaling that are currently in clinical trials (see discussion below) also will have such a robust therapeutic effect.

Glymphatic system and impaired AβO clearance

The recently discovered glymphatic system functions to remove metabolic waste, including soluble proteins, from the CNS [360]. The glymphatic system involves CSF inflow to the brain, which drives interstitial fluid to clear waste out of the brain. Recent studies in mice show that glymphatic activity decreases sharply during aging, resulting in decreased Aβ clearance from the brain [320]. Studies in AD mouse models show that this decreased Aβ clearance is due to oligomer formation [321], especially HMW AβOs [166], indicating that the size of larger AβOs may make it more difficult for the glymphatic system to clear them from the brain. Poor sleep quality, which is associated with dementia, might negatively affect the activity of the glymphatic system [340]. CSF levels of Aβ have been found to be increased significantly in insomnia patients [361]. Thus, the role of the glymphatic system in AD with regards to Aβ may be that its decreased activity leads to impaired AβO clearance, which may lead to further aggregation resulting in larger AβOs or insoluble amyloid plaques, both of which the glymphatic system cannot clear. Ultimately, repairing glymphatic activity may be another option for therapeutic targeting in AD treatment.

Blood-CSF barrier

The function of the blood-CSF barrier is to keep undesirable molecules and pathogens out of the brain. Several studies have shown that the integrity of the blood-CSF barrier is disrupted in AD [362], and recently, evidence has been presented that this disruption can be induced by AβOs, seemingly through increased expression and activity of matrix metalloproteinases [363]. Based on their data, the authors of this study hypothesize that AβO-induced breakdown of the blood-CSF barrier might be an early event in AD pathogenesis that would contribute to the enhancement of neuroinflammation. Therefore, early therapeutic inhibition of matrix metalloproteinases may decrease neuroinflammation in AD.

Newer AD models

There is growing consensus in the literature that Tg mice are not ideal models of AD, partly because they are based on genetic mutations present in only <5% of AD patients. Furthermore, although many therapeutics have ameliorated cognitive deficits and/or neuropathology in Tg mouse models, these same therapeutics have failed in clinical trials. The traditional Tg mouse models that express mutant forms of human APP and/or presenilins generally do not develop tau pathology unless they also express mutant forms of tau [364–367]. However, tau mutations are associated with other tauopathies, not AD. It has been argued that Tg mouse models actually simulate the asymptomatic phase of AD and therefore are telling us how to prevent AD, not cure AD [368]. Next-generation Tg

mouse models are being developed that accumulate Aβ without phenotypes related to Aβ overexpression, which may be unrelated to AD. It has been recommended that these models be used with the caveat that researchers consider the strengths and limitations of each model against the scientific and therapeutic goal of a prospective preclinical study [369]. There remains a call for more suitable models that recapitulate sporadic AD and more closely model human physiology.

Perhaps one of the most exciting AD model systems recently introduced is the NHP. NHPs have an APP sequence that is completely homologous to that of humans [370] and they develop plaques and tau pathology [370–372]. To speed up development of AD pathology, researchers introduced Aβ preparations containing fibrils into NHPs via i.c.v. injection. This resulted in microglial activation, neuronal loss, and tau phosphorylation [373, 374]. In a major advance for the AβO field, researchers from Brazil and Canada showed that i.c.v. injection of AβO preparations free of fibrils into NHPs induced fundamental features of AD pathology without development of Aβ fibrils and plaques [46]. The pathological features induced by AβOs in these NHPs included synapse loss, tau hyperphosphorylation, and activation of astrocytes and microglia. Most importantly, this research team recently reported that sustaining AβO injections for 12–18 months results in memory deficits and synapse loss [375]. This NHP model shows great promise as a superior AD model for therapeutic testing.

Another potentially powerful new AD model system comprises human induced pluripotent stem cells, or iPSCs. iPSCs derived from both familial AD and sporadic AD patients have been studied, and these AD-derived iPSCs show AβO accumulation, ER stress, oxidative stress, and tau hyperphosphorylation [376, 377]. These studies indicate that familial AD and sporadic AD iPSCs exhibit differential manifestations of ER stress [376] and $A\beta_{40}$ accumulation [377], indicating that different therapeutics may be effective for patient subsets. In 2015, an organoid human iPSC-derived system was developed, also termed a "3D human neural cell culture system" [378]. This iPSC system developed key events in AD pathogenesis, including extracellular aggregation of Aβ and accumulation of pTau. The De Strooper group recently created a novel chimeric model wherein human iPSCs were studied in a more natural environment, i.e., via transplantation into the brains of APP Tg immunodeficient mice [379]. These human neurons were able to differentiate and integrate into the mouse brain, express 3R/4R tau splice forms, show abnormal phosphorylation and conformational tau changes, and undergo neurodegeneration. Remarkably, transplantation of these human iPSCs altered gene expression, upregulating genes involved in myelination and downregulating genes related to memory and cognition, synaptic transmission, and neuronal projection. Therefore, human iPSC models are attractive AD models for their human origin and ability to integrate into mouse models, which are more easily utilized than NHP models.

Efforts are also being made at developing improved rodent models for AD. *Octodon degus*, a small rodent endemic to Chile, needs no genetic manipulation as its Aβ sequence differs from human in only 1 amino acid (H13R). Unlike mouse and rat Aβ, this Aβ sequence does form AβOs and this rodent also develops plaques and pTau with age [380, 381]. Tg rats are also being developed as AD models. The TgF344-AD rat expresses mutant APPsw and PS1ΔE9 genes and manifests age-dependent cerebral amyloidosis that precedes tauopathy, gliosis, apoptotic loss of neurons in the cerebral cortex and hippocampus, and cognitive disturbance [367]. These rats also exhibit pathological changes in the retina, including plaques and inflammation, e.g., microglial recruitment and complement activation [382]. This is interesting considering AβOs may also be involved in the pathogenesis of macular degeneration and glaucoma (see discussion below). Another Tg rat was developed using the Tg2576 mouse protocol. These rats exhibit cognitive deficits at 8–12 months, activated astrocytes in the brain, ThioS staining in the hippocampus and cortex, and elevated levels of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ [383]. Tg rats expressing the Swedish and Indiana APP mutations also exhibit elevated levels of these Aβ peptides in the CSF [384] as well as pre-plaque intracellular AβO-associated cognitive impairment [87]. There are indications that non-Tg rabbit, which expresses the human Aβ sequence, may also prove valuable for studies of AβO etiology [346].

The use of Drosophila as an AD model was reviewed recently [385]. Drosophila has homologues of human APP and tau. Other advantages of Drosophila for use as an AD model include low genome redundancy, which greatly simplifies the analysis of single gene disruption, short lifespan, and their low cost compared to mammalian models. Drosophila have been used to uncover or validate several pathological pathways or susceptibility genes

and have been readily implemented in drug screening pipelines. Interestingly, using a Tg Drosophila model expressing the Artic mutation, it was found that AD-like pathologies affected the circadian system in an age-dependent manner [386]. These Tg flies showed a dramatic degradation of circadian rhythms in tune with their reduced longevity and impaired climbing activity.

The use of *Caenorhabditis elegans* as an AD model was recently reviewed [387]. *C. elegans* is useful as an AD model as it has homologs of AD-related genes, including APP, tau, and PSEN1. *C. elegans* has complex biochemical pathways just like mammals, many of which are conserved. Its neuronal connectivity has already been established, making it a suitable model for learning and memory impairments [387]. Furthermore, *C. elegans* has a short lifespan, thereby speeding up study of Aβ accumulation, a process that can take months or years in other model organisms. To directly study the impact of the exact human $A\beta_{42}$ sequence, Tg worms also have been developed and these model organisms have been shown to accumulate AβOs and utilized to study AβO-directed therapeutics [388–391].

Therapeutics

Eliezer Masliah, the current head of the NIA's Division of Neuroscience, proposed multiple possible strategies for targeting the AβO pathogenic cascade in a 2014 commentary in *PNAS* (Fig. 9) [392]. He and co-author Cassia Overk proposed that therapeutics for AD might involve 1) directly clearing AβOs; 2) blocking AβO surface receptors; 3) interfering with AβO-induced signaling pathways; or 4) decreasing downstream effectors such as tau. The AD therapeutics currently in clinical trials are described in a systematic review of clinicaltrials.gov conducted in January 2017 by Cummings and colleagues [393]. Approximately half of the 105 agents currently in clinical trials are amyloid related. Figure 10 illustrates the mechanistic targets of many of the amyloid-targeted therapeutics. Some of the current efforts germane to AβO pathogenic mechanisms are discussed below.

1) Directly clearing AβOs or decreasing AβO production. This category comprises anti-Aβ immunotherapies, β-secretase (BACE) inhibitors, and anti-Aβ aggregation agents. About one quarter (27%) of agents in AD phase II clinical trials fall into this category. However, this category comprises more than half (57%) of phase III AD trials [393]. Several recent reviews discuss the progress of these

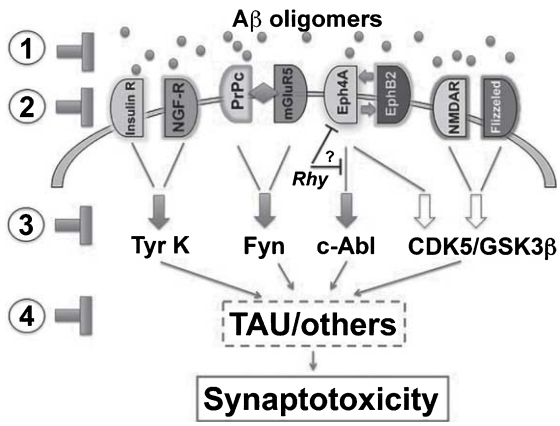

Fig. 9. Putative therapeutic targets of the AβO pathogenic cascade. Including: 1) AβOs themselves; 2) AβO receptors; 3) signaling pathways; or 4) downstream effectors such as tau. Reprinted with permission of PNAS from "Toward a unified therapeutics approach targeting putative amyloid-beta oligomer receptors" by Overk CR and Masliah E. This was published in *Proc Natl Acad Sci U S A*, 2014, 111(38): 13680-13681 [392].

Aβ-centric clinical trials and provide hypotheses for the failures. In 2014, Karran and Hardy reviewed the data reported at each phase of the drug discovery process for Aβ-targeting therapies and found significant gaps in the data in several cases [102]. They observe that target engagement was not established for most therapeutic agents analyzed, an issue also raised by Brody et al. [107]. In 2016, Selkoe presented an updated review of Aβ-targeted phase III clinical trials and concluded that they have failed because of improper patient selection, choice of agent, lack of target engagement, and/or dose or side effects unrelated to target engagement [5].

Anti-Aβ immunotherapies represent 8% of the phase II pipeline and 18% of the phase III pipeline. In 2014, Goure and colleagues of Acumen Pharmaceuticals proposed in their review of immunotherapeutics that current Aβ-directed therapies were failing due to lack of selectivity for AβOs; instead, they bind to monomeric or fibrillar Aβ, or both [101]. Monomers and fibrils are more abundant than AβOs in the AD brain, but less germane to nerve cell damage. The authors suggest that the affinity for monomers and/or fibrils by Aβ immunotherapies in clinical trials is why high doses are required for therapeutic benefit. Acumen, in partnership with Merck, has developed an antibody, known as ACU193, that is unique among Aβ immunotherapeutics in its selectivity for Aβ oligomers. ACU193 has greater than 500-fold selectivity for AβOs over fibrils [394] and greater than

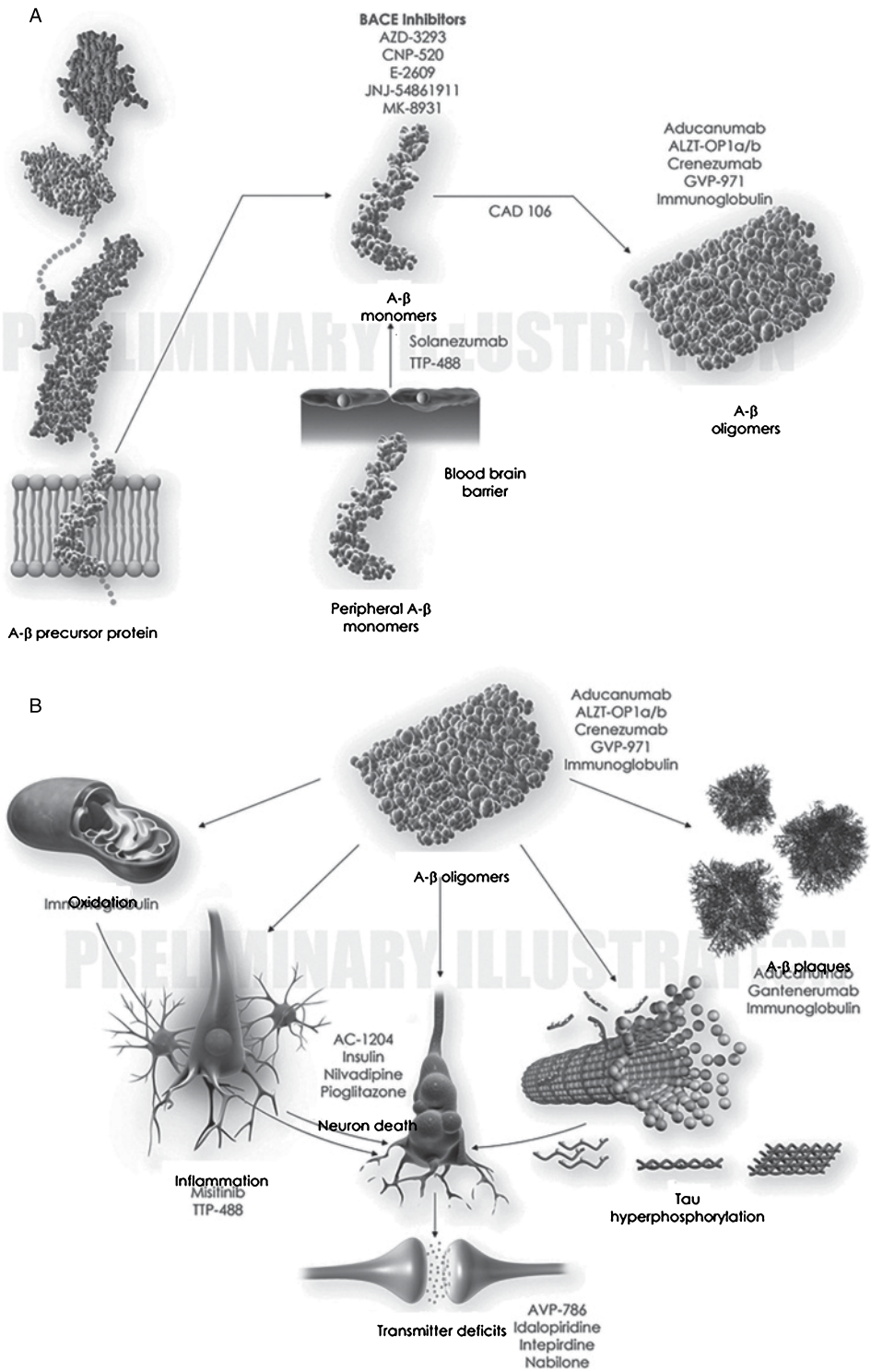

Fig. 10. Mechanisms of Aβ-targeting phase III drugs in AD clinical trials. Drugs inhibiting AβO formation (A) or downstream consequences of toxic AβOs (B). Reprinted from "Alzheimer's disease drug development pipeline: 2017" by Cummings J, Garam L, Mortsdorf T, Ritter A, and Zhong K. This was published in *Alzheimers Dementia (N Y)*, 2017, 3(3): 367-384 [393], under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License (CC BY NC ND) https://creativecommons.org/licenses/by-nc-nd/4.0/) [393].

2500-fold selectivity for AβOs over monomers [34]. Success of ACU193 in clinical trials would provide compelling evidence for the hypothesis that soluble AβOs are the primary toxins instigating AD pathogenesis.

What may be two examples supporting AβO-directed immunotherapies are Crenezumab and Aducanumab. Genentech reported at the 2017 Clinical Trials on Alzheimer's Disease (CTAD) conference that their Aβ immunotherapy Crenezumab had a 10-fold higher affinity for AβOs over Aβ monomers [395]. In immunoprecipitation experiments, its main target was AβOs that are, or dissociate into, SDS-stable dimers. It is of note that dimers themselves are not thought to be toxic [129]. Genentech reported that primary efficacy endpoints were not met for Crenezumab in two phase II trials (ABBY and BLAZE), although subgroup analysis showed greater reduction in cognitive decline in patients with mild AD given the higher dose of Crenezumab. They are currently enrolling patients in two phase III studies (CREAD and CREAD2), which will dose up to 4-fold higher.

Aducanumab (Biogen), an antibody that targets Aβ oligomers and fibrils, has shown in phase Ib trials a reduction of amyloid plaques in a dose- and time-dependent manner and, most importantly, a slowing of cognitive decline. Slowing of cognitive decline required the highest doses tested. However, these doses caused amyloid related imaging abnormalities [105]. While there is optimism that higher doses of these antibodies will result in greater therapeutic efficacy, a fully AβO-selective antibody may be essential for using low doses to avoid complications while still providing disease-modifying efficacy.

BACE inhibitors represent 6% of the phase II pipeline and 18% of the phase III pipeline. Supporting evidence for the therapeutic value of BACE inhibitors comes from the protective A673T APP Icelandic mutation in humans, which reduces BACE processing of AβPP [396, 397]. On the other hand, genetic deletion of BACE in mice causes many side effects, most notably the AD symptoms of neurodegeneration and memory dysfunction [398]. Since the systematic review conducted by Cummings and colleagues in January 2017 [393], Merck has halted its phase II and III trials of the BACE inhibitor verubecestat for lack of efficacy; a leading theory for this failure is the timing of treatment [399]. A recent success in AD mouse models, demonstrating beneficial effects on cellular, long-range circuitry, and memory impairment, has motivated researchers to start another clinical trial with a modified BACE inhibitor [400].

2) Blocking AβO receptors. The AβO receptors currently targeted in clinical trials are RAGE, the Sigma-2 receptor, calcium channels, and IR. Azeliragon (vTv Therapeutics), an inhibitor of RAGE, is currently in phase III clinical trials. RAGE has been identified as an AβO-targeted receptor, as reviewed previously [401]. This therapy did show a statistically significant slowing in cognitive decline in phase II trials, although it increased cognitive decline when tested at a higher dose [402]. A Sigma-2 receptor antagonist (CT1812; Cognition Therapeutics) is currently in phase II clinical trials. Sigma-2 receptors have been shown to participate in AβO binding to neurons and synaptotoxicity [204]. The Sigma-2 receptor antagonist blocks AβO binding to cultured neurons and improves cognitive deficits in AD mouse models [203]. In October 2017, the FDA placed CT1812 on fast track and in November, Cognition Therapeutics reported at CTAD that CT1812 was well tolerated at all doses tested [403]. Furthermore, it decreased levels of protein biomarkers including synaptotagmin-1, a marker of synaptic damage [404]. Nilvadipine, a calcium channel blocker (St. James' Hospital Ireland, Alzheimer Europe, Archer Pharmaceuticals) that is currently indicated to reduce blood pressure, has completed phase III clinical trials for AD, although no results yet have been reported. Nilvadipine has been reported to enhance Aβ clearance from the brain of AD mouse models and improve cognition; its putative mechanisms-of-action being inhibition of BACE, inhibition of RAGE-mediated Aβ brain influx, and/or facilitation of lipoprotein receptor (LRP-1)-mediated Aβ brain efflux [405].

The relationship between insulin resistance and AβOs in AD is discussed above. Therapeutically, insulin signaling may be a convenient target if the many treatments already developed for type 2 diabetes could be repurposed for AD [406, 407]. In hippocampal cell cultures, exogenous as well as astrocyte-secreted insulin and IGF1 displace AβOs bound to cell surfaces [73]. Diabetes drugs fall into five categories: intranasal insulin, incretins, dipeptidyl peptidase 4 (DPP-4) inhibitors, peroxisome proliferator activated receptor (PPAR-γ) agonists, and the common diabetes treatment metformin [406]. Intranasally delivered insulin has been shown to improve memory function in AD patients, although different studies have obtained inconsistent results as to whether this is effective in APOE E4-positive

AD patients [406]. The impact of intranasally delivered insulin on AD is currently being investigated in phase I and II clinical trials sponsored by Wake Forest University. Incretins are gastrointestinal hormones that stimulate insulin secretion and inhibit glucagon release in a glucose-dependent manner [406]. Liraglutide, an incretin analog, has been shown to reverse memory impairment, synaptic loss, and reduce plaque load in aged APP/PS1 mice [408]. Liraglutide is currently in phase II clinical trials (Imperial College London). DPP-4 inhibitors block degradation of incretins and have been found to improve memory function, reduce levels of Aβ and pTau, and decrease inflammation in AD rodent models [406]. However, no DPP-4 inhibitors are currently in clinical trials [406]. Activation of PPAR-γ induces the expression of multiple genes involved in the insulin signaling cascade, which improves insulin sensitivity in patients with type 2 diabetes [406]. Metformin, a very widely prescribed drug for diabetes [409], is currently in clinical trials to examine its effect both on aging in general and AD. On the other hand, it has been recently linked to an *increased* dementia risk in diabetes patients [410].

3) Interfering with AβO-induced signaling pathways. The 2014 commentary by Overk and Masliah [392] suggests the therapeutic targets in this category are kinases that are activated by the various AβO surface receptors. These kinases include the tyrosine kinases, Tyr K, Fyn, and c-Abl, and also CDK5/GSK3β. Fyn inhibition as a therapeutic strategy is based on the PrPc/mGluR5 pathway discussed earlier and has been reviewed recently [411]. An inhibitor of Src and Abl family kinases, AZD0530, is currently in phase II clinical trials (Yale University). AZD0530 (saracatinib) was previously used to treat cancer. Pre-clinically, it was found to reverse cognitive deficits in AD mice [412]. Another kinase inhibitor repurposed from cancer treatment, nilotinib (Tasigna®), is currently in phase II clinical trials (Georgetown University). Nilotinib targets the tyrosine kinase Abl and may aid in clearance of plaques and tangles through activation of autophagy [413, 414].

4) Decreasing downstream effectors such as tau. Tau-directed therapies, which could block the down-stream effects of AβOs [286, 415], represent 8% of the phase II pipeline and 4% of the phase III pipeline. Similar to Aβ-directed therapies, there are multiple mechanisms of action for therapeutic targeting of tau including inhibiting tau aggregation, decreasing tau hyperphosphorylation, reducing tau levels in the brain, and stabilizing microtubules [415]. LMTM (aka TRx0237; TauRx) is a small molecule derived from the dye methylene blue that has been shown to block tau aggregation *in vitro* and in tau Tg animal models [416]. Although it initially showed no clinical efficacy when tested as a combination therapy [416], it recently showed an ability to improve cognition and decrease rate of brain atrophy when tested as a monotherapy in phase III clinical trials [417]. No significant efficacy findings have been reported yet for other tau-directed therapies.

Combination treatments

Given the complex neuropathology of AD and the lack of effective biomarkers for sporadic AD, it may be the case that multi-factorial, combination treatments will provide the greatest efficacy in AD treatment. This is a sentiment shared by many in the field [5, 66, 106, 418]. In October 2017, Amylyx Pharmaceuticals received a grant from the Alzheimer's Association and the Alzheimer's Drug Discovery Foundation to conduct phase II clinical trials with the combination therapy AMX0035 (Alzheimer's Association). AMX0035 is a combination of two compounds that synergistically block mitochondrial and ER stress. Preclinical studies show that this combination protects brain cells from inflammation and oxidation in models of amyotrophic lateral sclerosis, AD, and mitochondrial diseases [419]. ALZT-OP1 (AZTherapies) is a combination of two small molecule drugs, cromolyn, an asthma drug, and ibuprofen. Cromolyn was found to inhibit Aβ aggregation *in vitro* and reduce soluble Aβ in the brain *in vivo* [420]. The intended effect of ibuprofen in the combination therapy is to reduce neuroinflammation. ALZT-OP1 is currently in phase III clinical trials. It is likely that many more combination therapies will arise in the future.

AD prevention: Diet, exercise, and mental/social engagement

One review article states that although it is difficult to make conclusions regarding diet as an AD risk-factor due to the difficulty in analyzing eating patterns, there does seem to be clear evidence that diet influences AD. Protective foods identified include fish, fruit, coffee, and wine. There is also evidence that a diet high in saturated fats may increase AD risk [421]. In a Tg mouse AD model, high cholesterol

promotes earlier buildup of AβOs [350]. A systematic review and meta-analysis by researchers at the Mayo Clinic found that a higher adherence to the Mediterranean diet is associated with a reduced risk of developing mild cognitive impairment (MCI) and AD and a reduced risk of progressing from MCI to AD [422]. Vitamin B may also have a positive effect [423]. To more directly test the impact of diet on AD risk, participants are currently being recruited for a clinical trial to determine the effect of saturated fat and glycemic index on cognition in older individuals with or without an APOE E4 genotype (sponsored by the University of Washington). APOE4 is known to affect the presence and impact of AβOs [424–430]. Recruitment is underway also for clinical trials testing Genistein, a dietary supplement that has been found to have antioxidant and neuroprotective effects on AD and increases PPAR-γ levels, which results in increased APOE expression and Aβ degradation (Fundación para la Investigación del Hospital Clínico de Valencia). The omega-3 fatty acid DHA, which protects against AβO-instigated dendritic spine loss [431], shows potential to decrease AD incidence in APOE4 carriers [423, 432] and is under clinical investigation (http://clinicaltrails.gov). In addition to the potential for diet to modify AD risk, it is well recognized that physical activity also modifies risk [433]. In AD mouse models, exercise decreases AβO levels and increases cognitive performance [434–437]. Furthermore, evidence has been found for mental/social engagement modifying AD risk and AβO accumulation in a mouse model [438] and in humans [439–441]. Therefore, it is encouraging that some cases of sporadic AD may be delayed or even prevented by modifiable lifestyle factors.

AβOs as biomarkers: can AβOs provide metrics to assess experimental drug efficacy and ultimately give a diagnostic to indicate a patient should start AD treatments?

Newly emerging approaches have begun to focus on therapeutic targeting of ABOs, and not amyloid plaques, as AβOs are the form of Aβ that instigates the neural damage leading to AD. A powerful metric for the efficacy of these new approaches to disease-modifying therapeutics would be to monitor a patient's AβO levels.

There are two big challenges to using AβO levels as a biomarker. First, there is a need for extraordinary sensitivity. Second, there is a need to discriminate oligomeric Aβ from the other, much more abundant but chemically similar forms of the peptide. A uniquely sensitive and specific AβO immunoassay, capable of attomolar quantitation, initially showed that median AβO levels in AD CSF were 10-30-fold higher than in non-AD controls [32]. Although powerful, this assay was not practical, and it has not been explored for clinical use. Since then, other groups have utilized various immunoassay platforms and Aβ-targeting antibodies with varying results. A team at Merck using the AβO-specific antibody ACU193 (see Therapeutics section above), found a significant 3-5-fold increase in AβOs in AD CSF compared with aged-matched controls [34]. On the contrary, Jongbloed and colleagues found that CSF AβO levels decreased from non-dementia to MCI to AD [165]. Another study found no significant difference between AβO levels in AD CSF and controls. However, this study found a small, but significant increase in AβOs levels in MCI compared to controls, suggesting an early rise in AβO levels followed by a later decrease [35]. Most recently, an AβO-specific plasma assay was able to differentiate AD from controls with 78.3% sensitivity and 86.5% specificity, finding AβOs elevated in AD plasma [442]. CSF/plasma measurements of whole-AβO populations do not seem to be diagnostically useful at present due to inconsistent results, but the possibility remains that an assay targeting specific populations of AβOs may be more useful [33, 35, 443–445]. Indeed, a recent study showed that an assay targeting HMW AβOs could be used to monitor efficacy of an anti-amyloid therapeutic [446]. This finding is consistent with immunoassays showing therapeutic efficacy in a mouse model correlated with reduction in a pool of putative type 1 AβOs specifically recognized by the NU4 monoclonal antibody [120].

An alternative to measurements of AβOs in CSF or blood comes from new technologies for AβO imaging. The Pronucleon™ platform from Adlyfe consists of series of engineered peptides that provide a unique readout when associated with beta-rich fibers and AβOs. There are indications that it can be used for pre-plaque imaging [447]. Another technology is the development of magnetic resonance imaging (MRI) [96] and positron emission tomography (PET) [199] probes using modified antibodies that have high selectivity and affinity for AβOs. These have been shown to discriminate AD Tg mice from non-Tg littermates and to discriminate human AD brain slices from non-AD specimens. The availabil-

ity of humanized AβO-specific antibodies makes it likely that these probes will soon be ready for clinical testing [101].

In the future, it is foreseeable that scheduled clinical tests of AβO levels could provide an indicator of whether a patient should begin an appropriate AD treatment. Monitoring levels after diagnosis and treatment would provide an initial indicator of how well a patient is responding to a therapeutic. Use of AβO levels as an AD biomarker would thus be akin to monitoring glycated hemoglobin by A1C levels for diabetics.

Other dysfunctions/degenerative neural disorders linked to AβOs?

As discussed above, TBI and diabetes may be etiological factors in the buildup of AβOs. In addition, it may be the case that once accumulated, AβOs may contribute to further pathogenic consequences in these diseases such as insulin resistance (see discussion above), dementia, or chronic traumatic encephalopathy (CTE). AβOs, specifically, have not yet been implicated in CTE, but CTE is associated with repetitive brain injury and amyloid plaques have been observed in CTE brain tissue [448]. Additional disorders that have been linked to AβOs are inclusion body myositis, glaucoma, and macular degeneration. Inclusion body myositis is the most common progressive muscle disease of patients above the age of 50. Forms of Aβ, including AβOs, are known to accumulate in the muscle fibers of diseased patients [449]. Further evidence implicates AβOs in glaucoma and macular degeneration. AβOs have been found to contribute to apoptosis of retinal ganglion cells in glaucoma [450]. Intravitreal injection of AβOs in rat induces molecular changes associated with apoptosis in the rat retina. Apoptosis is hypothesized to take place in macular degeneration according to bioinformatics analysis [451]. AβOs, through RAGE, upregulate VEGF, which stimulates neovascular age-related macular degeneration [452]. Structures reactive with the OC antibody have been found in drusen, a hallmark of eyes affected by macular degeneration [453]. The OC antibody is well characterized and binds to fibrillar type 2 AβOs [148]. In fact, a modulator of Aβ aggregation (MRZ-99030) is neuroprotective with therapeutic treatment in animal models of glaucoma and macular degeneration [454]. However, much more evidence is needed to understand the role of AβOs in these neural disorders.

CONCLUDING REMARKS

As evidenced by the increasing number of publications concerning AβOs in the past 5 years, and the consistency in data supporting a toxic role for AβOs, the AβO hypothesis for AD pathogenesis has garnered considerable support and acceptance. Accordingly, the number of AβO-targeting therapeutics in the AD pipeline has begun to increase. We believe that this emerging interest in AβO targeting will prove beneficial to the treatment and diagnosis of AD. These efforts potentially can extend to a broader proportion of the population, given the evidence for a role for AβOs in other diseases in addition to AD. Ultimately, for these efforts to result in therapeutic and diagnostic benefits, further advances in AβO structure-function studies are needed. Continued investment into this and other research involving AβOs will enable the closing of critical gaps, thereby paving a smoother and shorter path from bench to bedside.

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Role of Amyloid-β and Tau Proteins in Alzheimer's Disease: Confuting the Amyloid Cascade

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Abstract. The "Amyloid Cascade Hypothesis" has dominated the Alzheimer's disease (AD) field in the last 25 years. It posits that the increase of amyloid-β (Aβ) is the key event in AD that triggers tau pathology followed by neuronal death and eventually, the disease. However, therapeutic approaches aimed at decreasing Aβ levels have so far failed, and tau-based clinical trials have not yet produced positive findings. This begs the question of whether the hypothesis is correct. Here we have examined literature on the role of Aβ and tau in synaptic dysfunction, memory loss, and seeding and spreading of AD, highlighting important parallelisms between the two proteins in all of these phenomena. We discuss novel findings showing binding of both Aβ and tau oligomers to amyloid-β protein precursor (AβPP), and the requirement for the presence of this protein for both Aβ and tau to enter neurons and induce abnormal synaptic function and memory. Most importantly, we propose a novel view of AD pathogenesis in which extracellular oligomers of Aβ and tau act in parallel and upstream of AβPP. Such a view will call for a reconsideration of therapeutic approaches directed against Aβ and tau, paving the way to an increased interest toward AβPP, both for understanding the pathogenesis of the disease and elaborating new therapeutic strategies.

Keywords: Amyloid-β peptide, amyloid-β protein precursor, oligomers, synaptic dysfunction, tau

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Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by dementia, defined as a deficit of memory function and at least one other cognitive domain (language, praxis, gnosis, executive function, judgment, and abstract thought) as well as functional impairment, without alteration of the state of consciousness. In the last decades, AD has gained rising attention for its growing prevalence in aging populations, with 46.8 million people affected by the pathology worldwide, a number expected to increase up to 74.7 million in 2030 and 131.5 million in 2050. Besides representing a serious health and social problem, the disease causes exorbitant costs for the healthcare system estimated as 604 billion dollars in 2010 that represented a 35.4% increase in only 5 years [1, 2]. Despite the numerous efforts to counteract the disease, no therapies have so far proven to prevent AD onset or progression.

To date, data from thousands of basic, pre-clinical, and clinical studies have identified amyloid-β peptide (Aβ) and tau protein as the key actors in the pathophysiology of AD, mainly because of their deposition in the characteristic histopathological brain lesions, the senile plaques for Aβ and the neurofibrillary tangles (NFTs) for tau, and the increase of their soluble forms in the brain of AD patients. However, therapeutic approaches aimed to decrease Aβ levels that have been attempted so far, have failed. Similarly, tau-based clinical trials have not yet produced positive findings. The overall goal of this review is to provide a critical assessment of the literature on mechanisms underlying disease occurrence and progression. Specifically, we will revisit studies on Aβ and tau, as well as on their interaction, challenging the amyloid hypothesis that has dominated the AD field in the last 25 years. This hypothesis establishes Aβ as the *primum movens* in a cascade of pathological events that places tau downstream of Aβ. According to this hypothesis, once tau pathology has ensued, therapies against Aβ would no longer work because the disease would progress independently [3]. We propose rearranging the intricate puzzle of AD pathogenesis by placing soluble forms of Aβ and tau in parallel and upstream of amyloid-β protein precursor (AβPP), which would permit the peptides to exert their toxic functions. Such a view will call for a reconsideration of the reasons for the failure of anti-Aβ therapies, no longer attributable to the fact that they were started after triggering of tau pathology, necessarily changing the approach to studies on the etiopathogenesis of AD and paving the way for new therapeutic strategies.

AMYLOID-β PEPTIDE AND ALZHEIMER'S DISEASE: MORE THAN ONE CENTURY OF RESEARCH

Aβ derives from a complex cleavage of AβPP, a type I single-pass transmembrane protein constituted by 639–770 amino acids in humans, and highly expressed in the central nervous system where it exerts a variety of physiological functions [4]. AβPP is initially cleaved by α-secretase or β-secretase, generating soluble and carboxyterminal fragments (CTF). α-secretase activity leads to the formation of sAβPPα and CTF83, whereas β-secretase generates sAβPPβ and CTF99. Then, γ-secretase intervenes, further cleaving CTF83 and CTF99, generating the intracellular peptide AICD/AID (amyloid intracellular domain) and a small p3 peptide from CTF83, and AICD/AID and Aβ from CTF99. Based on this biochemical processing, the cascade initiated by α-secretase has been considered neuroprotective when compared with the β-secretase cleavage, leading to the amyloidogenic cascade and the formation of Aβ [5]. Based on the γ-secretase site of cutting, different isoforms of Aβ can be generated, composed of 38–43 amino acids. $A\beta_{40}$ is the predominant species, whereas $A\beta_{42}$ is present at lower concentrations but has received more attention in the AD field due to its high propensity to form aggregates. However, in the brain of AD patients, $A\beta_{38}$ and truncated forms at N-terminal region, i.e., $A\beta_{15}$, $A\beta_{16}$, and $A\beta_{17}$, have been also detected [6]. Aβ is undoubtedly the most studied protein in AD and its putative role in the pathogenesis of the disease has oriented drug development and clinical trials for several decades. But how and why did the AD amyloidogenic theory emerge?

From a historical perspective, it was at the beginning of the last century when Alois Alzheimer and other European neuropsychiatrists, e.g., Gaetano Perusini, attributed a nosographic identity to a form of "mental" disorder characterized by memory loss, hallucinations, and disorientation. At that time, the most influent personalities in psychiatry, Sigmund Freud and Emilin Kraeplin, fervently disputed on the origin of psychiatric illness, respectively emphasizing the role of the psyche or of organic and genetic factors. The mind/brain diatribe led several scientists to seek for the "material" causes of mental diseases. In this context, Alzheimer and Perusini, strongly supported by Kraeplin, observed that the psychiatric symptoms of dementia could be correlated to peculiar histological lesions in postmortem brains. In the

autopsy of the first described AD patient, Auguste Deter, cortical atrophy, neurons filled with neurofibrils, and extracellular miliary foci of an unknown substance were observed. After Alzheimer's death, research studies on the disease were few until the 1980 s, when epidemiological studies revealed an increase of patients affected by primary dementia. It was during these years that key discoveries were made, fated to influence research in the field until today. Based on Alzheimer's histological descriptions, Aβ and tau were recognized as the main components of extracellular foci (senile plaques) and intracellular neurofibrils (NFTs), respectively [7–9]. In the same period, the first genetic mutation linked to dementia was identified on chromosome 21 coding for the AβPP [10]. This autosomal dominant disease was responsible for early onset AD (EOAD) characterized by high levels of Aβ. Other genetic mutations were identified in Familiar Alzheimer's disease (FAD), involving genes responsible for Aβ production such as presenilin 1 (PS1) on chromosome 14, which mutation is the most common cause of EOAD, and presenilin 2 (PS2) on chromosome 1. Consistent with these findings, the presence of AD-like pathology in patients affected by Down's syndrome, due to a trisomy of chromosome 21, reinforced the idea that the increase of Aβ played a major role in AD pathogenesis. Based on these data, in 1995 the first mouse model of AD carrying an AβPP mutation was engineered [11] and, over time, different models for pre-clinical studies have been generated based on the most common mutations observed in FAD [12].

These findings contributed to the excitement around the "Amyloid Cascade Hypothesis" [13–15], recognized as the pathogenic mechanism underlying AD. Because insoluble fibrils of Aβ were present in AD plaques, and could be formed *in vitro* from synthetic Aβ, they have dominated the scene until a fundamental breakthrough confirmed by several *in vitro* and *in vivo* studies indicated that soluble forms of Aβ were also present in the brain [16, 17]. Aβ soluble aggregates range from monomers to oligomers (molecular aggregates consisting of a few monomer units) and pre-clinical studies confirmed that dimers, trimers, tetramers, dodecamers, and high molecular weight oligomers were all able to induce neurotoxic effects as well as to induce an immediate impairment of synaptic plasticity, and in particular of hippocampal long-term potentiation (LTP), thought to be the electrophysiological correlate of memory (for a review on the role of Aβ oligomers, see [18]). Moreover, Aβ oligomer presence in human cerebrospinal fluid (CSF) could be already recognized decades before AD onset [19]. These data led to the formulation of another theory, the "Oligomer Hypothesis" [20, 21], according to which Aβ oligomers but not monomers or fibrils were responsible for synaptic dysfunction and memory loss in AD [22, 23]. This further influenced AD drug discovery so that new therapies aimed at specifically targeting Aβ oligomers were developed in addition to those clearing Aβ plaques.

Unfortunately, while the "Oligomer Hypothesis" is still a matter of investigation, and data are being gathered to test the grounds of its premises, the clinical failure of most of the anti-Aβ drugs has strongly destabilized this concept. Clinical trials to date show that, despite successful results obtained in animal models of AD, anti-Aβ drugs have not yet been shown to modify cognition in humans although they might be able to reduce plaque or amyloid burden. So far (based on Medline database search and ClinicalTrials.gov): 1) active immunization (i.e., AN-1792, CAD-106, and vanutide cridificar) have not proven effective and several side effects were reported; 2) passive immunization with monoclonal antibodies bapineuzumab, solanezumab, crenezumab, and gantenerumab have not yet succeeded, and although a recent clinical trial with aducanumab has shown a dose-dependent reduction of Aβ plaques, the study was not sufficiently powered to detect clinical changes and the drug is undergoing further investigation [24]; and 3) a number of clinical trials with drugs aimed at preventing Aβ formation by inhibiting β- or γ- secretases have also failed or were interrupted; among these, the γ-secretase inhibitors semagacestat and avagacestat did not show efficacy, and actually induced mild worsening in cognition and severe side effects, whereas the EPOCH trial with the newest β-secretase inhibitor verubecestat was stopped for the lack of any positive effect. Notwithstanding these discouraging results, several scientists are still developing anti-Aβ therapies, convinced that the failure of Aβ tailored drugs might relate to the particular drugs chosen, inadequate dosage, or the fact that treatment was started in a late phase of the disease when Aβ-induced damage cannot be reversed.

This review is written, in turn, with the belief that a careful evaluation of the knowledge in the AD field is due prior to further investing resources with anti-Aβ therapies. Evidences that have been underestimated

for a long time are now gaining ground, questioning the way in which the actual role of Aβ in AD pathogenesis is currently thought. First, late onset AD (LOAD), representing 95% of AD cases, is not linked to genetic anomalies leading to a direct overproduction of Aβ, as in FAD, although the phenotype might be comparable. However, pre-clinical studies on AD mouse models have been almost entirely performed on mice presenting FAD-like mutations leading to an increase of Aβ. Second, we know since the 1990 s that there is no correlation between Aβ deposition and clinical degree of dementia among affected individuals [25–28], and plaques might occur in the brains of individuals with no sign of dementia [27, 29, 30]. Third, recent studies have suggested that plaque formation might be a reactive process [31] with a protective role by decreasing oligomer levels [32]. Fourth, a vast literature claims that Aβ exerts a physiological role in the CNS interfering with neuronal growth, neurotransmitter release, synaptic function, and memory formation [33, 34]. Indeed, our group and others have previously demonstrated that administration of low concentration of oligomeric Aβ positively modulate synaptic function [35–37] and, conversely, blocking endogenous Aβ in the healthy brain resulted in an impairment of synaptic plasticity and memory [36, 38]. Finally, even Aβ concentration *per se* has become a relative concept, as the persistence of a low picomolar Aβ concentration in extracellular fluids provides for detrimental outcomes in synaptic plasticity [39]. In conclusion, taking into account almost one century of research, it emerges that the Aβ model of AD is insufficient [40, 41] and needs to be reconsidered [34].

A REVALUED PLAYER IN ALZHEIMER'S DISEASE PATHOGENESIS: TAU PROTEIN

As described above, the intricate story of Aβ and tau began with the brain of Auguste Deter, but most of the research efforts have been directed toward Aβ. Recently, the discontent generated by too many anti-Aβ therapy failures has induced several groups to re-focus on tau.

Tau is a microtubule-associated protein originally described as a heat stable protein essential for microtubule assembly and stabilization [42]. It is present in the human brain in six main isoforms, deriving from the alternative splicing of exons 2, 3, and 10 of microtubule-associated protein tau (MAPT) gene. This process appears to be of particular interest for exon 10 splicing which determines the presence of tau isoforms containing 3- (3R) or 4-repeats (4R) of a ~32 amino acid sequence in the microtubule binding domain (MBD) [43]. Moreover, the splicing process of exons 2 and 3 determines the number of 29-residue near-amino-terminal inserts which results in isoforms containing 0, 1, or 2 inserts (0N, 1N, 2N) [44]. Both R and N repeats are capable of microtubule-binding and assembly-promoting activity, whereas the flanking regions are more likely involved in binding processes [45, 46]. In the last decades, many studies have demonstrated tau physiological involvement at different subcellular localizations: 1) at axonal level, by regulating axonal elongation, maturation and transport [47–50]; 2) in dendrites, participating in synaptic plasticity [51, 52]; and 3) in nucleus, maintaining the integrity of genomic DNA, cytoplasmic and nuclear RNA [53, 54].

From a functional point of view, tau can be divided in four different regions consisting of a N-terminal domain, a proline-rich domain, a MBD, and a C-terminal domain [3, 55, 56]. The N-terminal domain is rich with negative charges devoted to separation of different microtubules by electrostatic repulsion when tau is bound to a certain microtubule [46, 57, 58]. Interestingly, the C-terminal domain, besides its key role in regulation of microtubule polymerization induction and interaction with plasma membranes [59–62], creates an overall asymmetry in the molecule contributing to this microtubule spacing function thanks to its neutral charge. The proline-rich domain and the MBD with their multiple aminoacidic acceptor residues are more involved in interactions with different signaling proteins, which can be scaffolded by tau or can modify tau conformational status and activity itself [3].

The presence of multiple binding sites confers to tau many interaction possibilities in regards to cell signaling. The flanking region of MBD contains the majority of phosphate acceptor residues, and the phosphorylation of these sites is relevant for regulating microtubule polymerization [63–66], regulation of axonal transport [67] and neurotransmitter receptors [68, 69], interference with vesicles trafficking [70] and apolipoprotein E [71], interaction with Src-family kinases [62, 72–75], and many others [3, 55, 56].

The multiple roles of tau in neuronal physiology have been widely studied and, undoubtedly, a fine regulation is needed to maintain tau structure and function. Accordingly, a wide range of neurodegenerative disorders known as tauopathies

have been recognized and classified with respect to the predominant species of tau that accumulates: 1) 3R tauopathies (i.e., Pick's disease); 2) 4R tauopathies (i.e., corticobasal degeneration and progressive supranuclear palsy); and 3) 3R + 4R tauopathies (i.e., AD) [43].

Biochemical studies have demonstrated that deposition of insoluble tau aggregates in NFTs depends upon a dysregulated phosphorylation process of the flanking regions of tau. In fact, while two phosphates per molecule of tau are normally present [76], analysis of tau from AD brains has revealed the presence of about eight phosphates per molecule of tau [77]. For this reason, tau phosphorylation abnormalities have been linked to misfolding and deposition of the protein in the diseased brain [78]. Although tau has been defined as a "natively unfolded" protein with a low tendency to aggregation [79], phosphorylation of certain residues or detachment from microtubules [79–81] might change its conformational status and consequently its aggregation propensity. However, the undefined structure of tau in solution has precluded crystallographic analyses leaving a lack of knowledge about the protein structure [82]. Moreover, notwithstanding electron microscopic visualization of tau bound to microtubules demonstrated a linear alignment lengthwise to the protofilament ridges, the protein structure keeps holding a disordered state [83, 84]. Interestingly, when in a solution, tau spontaneously tends to modify its conformation in favor of a paperclip-like structure that might prevent its aggregation [55, 82], unlike Aβ that has a high tendency to aggregate in a solution due to its biochemical properties. Thus, alterations of tau (i.e., hyperphosphorylation, truncated forms) could inhibit the constitution of the paperclip-like structure leading to paired helical filament (PHF) and NFT formation [85]. In this context, tau hyperphosphorylation has been widely studied and the sequence hyperphosphorylation→PHFs→NFTs linked to AD, even if it is unlikely to represent by itself the main pathogenic event for several reasons. First, tau phosphorylation has been demonstrated to be responsible for aggregation only when occurring at certain residues [86], whereas in other sites it has the opposite effect thus preventing aggregation [80]. Moreover, tau hyperphosphorylation is not a prerogative of AD, since it occurs in several other conditions such as hypothermia [87], starvation [88], chronic stress [89], and anesthesia [90, 91].

Interestingly, the amount of PHFs and NFTs is slightly related to the severity of neuronal damage and cognitive impairment in humans. Experiments on regulatable mouse models of tauopathy demonstrated that a variant of human tau with the pro-aggregant mutation ΔK280 developed synaptic and memory impairment as well as tau hyperphosphorylation and pre-tangle formation. However, when the pro-aggregant tau was turned off, synaptic deficit was rescued even if insoluble tau was still present [92]. Other studies on transgenic mice expressing mutant tau (P301L mutation), which could be suppressed with doxycycline, demonstrated that behavioral impairment and neuronal loss were recovered when suppressing transgenic tau, whereas NFTs accumulation continued [93]. Moreover, in the P301S model of tauopathy, synaptic damage and cognitive impairment occurred before the emergence of NFTs [94]. Some authors also reported that, *in vitro*, abnormally phosphorylated tau can sequester normal tau into tangles of filaments, leading to the hypothesis that tau accumulation into PHFs might initially be neuroprotective until it starts compromising neuronal function as a space-occupying lesion [95].

The observations that synaptic and memory impairment is not mediated by NFTs, and that insoluble deposition of tau might be a compensatory protective mechanism suggested that synaptic failure might be sought in soluble oligomeric species of tau, resembling the "Oligomeric Hypothesis" already formulated for Aβ. Soluble tau was found to be most acutely toxic in animal models of tauopathy [93, 94, 96]. Most importantly, increases in granular tau oligomer levels occur before NFTs form and before individuals manifest clinical symptoms of AD, suggesting that increases in tau oligomer levels may represent a very early sign of brain aging and AD [97]. We have recently demonstrated that an acute exposure to tau oligomers (but not monomers) both *in vitro* and *in vivo* is detrimental to LTP and memory [98]. Noteworthy, this toxic effect was exerted by a different preparation of oligomeric tau, i.e., recombinant tau 4R/2N, tau derived from AD patients, tau derived from hTau mice [98]. These results are in agreement with other observations reporting that tau oligomers 1) impair synaptic function and memory in wild type mice [99], 2) correlate with cognitive impairment in rTg4510 mice [100], and 3) accelerate pathology in hTau mice [101].

Pre-clinical findings have been confirmed by studies on humans showing the increase of oligomeric forms of tau in the brain of AD patients compared to controls, occurring before NFT formation and

clinical symptoms [97]. Interestingly, tau oligomers have been also found in other tauopathies such as progressive supranuclear palsy, dementia with Lewy bodies, and Huntington's disease [101–103]. In AD brain, homogenates of tau dimers are also markedly elevated, suggesting that tau aggregation might be a hierarchical process that passes through distinct phases, i.e., monomers, dimers, oligomers, pre-tangles, and tangles [104]. Notably, the time-course leading from monomers to insoluble deposits is comparable to that already described for Aβ, with soluble forms of the peptide increasing in an initial phase of the disease.

Based on the findings described above and considering the urgent need to find more valuable biomarkers for an early diagnosis, the possibility of detecting tau oligomers in CSF of living patients is appealing. Hence, we have conducted a pilot study to verify that soluble aggregated forms of tau are detectable outside neurons in the CSF of living people and therefore they are not necessarily the byproduct of pathological alterations occurring in postmortem evaluations. We characterized tau immunoreactivity by western blot in CSF samples [105] from a cohort of 11 patients with probable AD and 11 healthy control (HC) individuals at the time of harvesting CSF (Table 1). High molecular immunoreactive species for total tau were observed in all the samples (Fig. 1A, B). However, a significant change in intensity of different bands was found, with an increase in the high molecular weight bands, presumably corresponding to oligomers, coincident with a decrease at 31–38 kDa in AD patient CSF compared to HC (Fig. 1A, B).

Interestingly, when we dissociated tau by treating the CSF samples with the reducing agent beta-mercaptoethanol (βME) to disrupt the thiol bonds between tau molecules, the signal intensity of high molecular weight tau immunoreactivity became undetectable, whereas a clear signal was present for monomeric tau, suggesting that the presence of oligomers was linked to disulfide bridges involving tau molecules (Fig. 1C). This study leads to important considerations. First, the possibility to evaluate the presence of extracellular oligomeric tau in clinical lumbar CSF specimens could be useful as a possible early biomarker of the disease, in agreement with other findings [102, 106]. Second, the observations that tau oligomers are also present in HCs and that monomers/oligomers are differently distributed in AD and control CSF suggest that the biological significance of tau species should be further investigated. These aspects should be taken into account when designing new drugs targeting tau to avoid the same issues already experienced with anti-Aβ treatments.

Table 1
Patients characteristics. The diagnosis of probable AD or control for healthy individual was done according to the NINCDS-ADRDA Alzheimer's Criteria

Patients #	Diagnosis	Age	Gender	MMSE
13	HC	65	W	30
14	HC	64	W	27
15	HC	69	W	27
16	HC	57	W	27
17	HC	66	M	29
18	HC	55	M	28
19	HC	73	M	26
20	HC	58	W	30
21	HC	83	M	28
22	HC	73	W	28
24	HC	79	W	29
2	AD	76	M	18
3	AD	72	M	28
4	AD	58	M	23
5	AD	68	M	17
6	AD	66	M	25
7	AD	54	M	25
8	AD	81	M	26
9	AD	71	M	27
10	AD	69	M	24
11	AD	64	W	25
12	AD	68	M	26

Diagnosis was determined after full neurological history and examination including testing of mental status. All diagnoses were made by an experienced neurologist, psychiatrist, or a consensus conference including neurologists and neuropsychologists. Cerebrospinal fluid samples were banked at Columbia University, under protocols approved by the Columbia University and New York State Psychiatric Institute Institutional Review Boards. HC: range 55–83 years, average: 67.45 ± 2.72; probable AD: range: 54–81 years, average: 67.91 ± 2.29 years. MMSE, Mini-Mental State Examination.

Notwithstanding the increase of tau oligomers in the AD brain and CSF, drugs aimed at inhibiting tau aggregation or dissolving existing aggregates, i.e., methylthioninium chloride and its second-generation derivatives such as TRx0237, have not been proven efficacious in clinical trials. A Phase II study with TRx0237 was terminated after a few months for "administrative" reasons, whereas Phase III studies have reported negative results on cognitive improvement (see clinicaltrials.gov for details). However, it is not clear whether these drugs actually inhibit tau aggregation in humans. Also, this makes us wonder whether the increase of tau oligomers in AD patients should be better considered as a pathogenic marker of the disease rather than a target of therapeutic strategies.

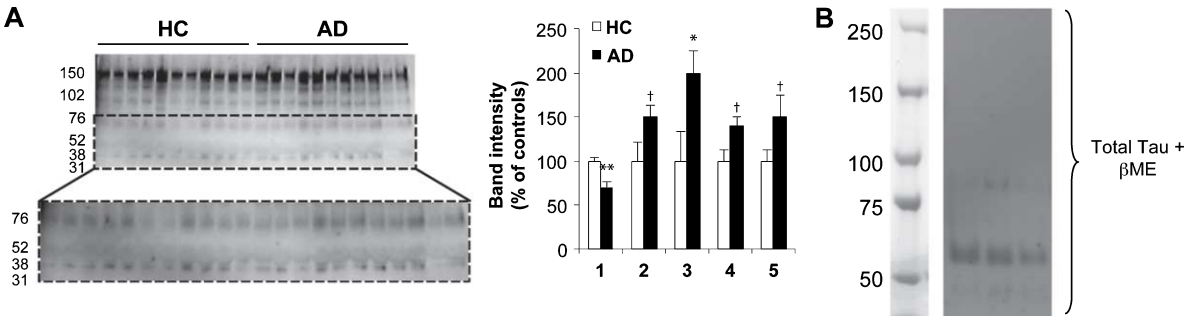

Fig. 1. Oligomeric tau is present in the CSF of AD patients and healthy individuals. A) Western blot showing total tau levels in CSF samples of healthy individuals (HC) and probable AD patients (higher magnification view of the lower molecular weight bands on the lower part of the panel. Different band intensity is quantified on the right graph (31–38 kDa: $p = 0.009$, 50–54 kDa: $p = 0.003$, 74–78 kDa: $p = 0.04$, 100–104 kDa: $p = 0.002$ and 120–150 kDa: $p = 0.003$). CSF specimens from subjects listed in Table 1 were thoroughly mixed, de-identified, and underwent one freeze–thaw cycle before standard aliquoting in 1.5 ml portions in polypropylene screw-cap tubes and storage at –80°C. To verify the oligomerization status of tau, we ran samples on western blots. Immunoreactivity toward total tau was measured in each of the CSF aliquots. Equal amounts of protein (8 μg) were fractionated by Tris-Acetate gradient gels (3–8%) and transferred to nitrocellulose membranes (Millipore). Tau immunoreactivity was detected using anti-total tau polyclonal antibody (1:2000; Epitomics). Immunoblot data were quantified by measuring the band intensity using imaging software (NIH ImageJ). Statistical analyses were performed by ANOVA plus *post-hoc* multiple comparisons test using Prism (GraphPad) software. B) Immunoreactivity for total tau in samples from probable AD patients reduced with β-mercaptoethanol (βME). βME zeroed the high molecular weight signal revealed by tau antibodies while intensifying the signals in the monomeric range.

Aβ AND TAU OLIGOMERS: A GAME AT THE SYNAPSE RESULTING IN MEMORY IMPAIRMENT

How do Aβ and tau induce memory loss? According to most of the studies, the answer should be sought at the synapse. Although cortical atrophy and synaptic loss have been reported in AD brains, mainly due to a structural damage imputable to plaques and tangles in a later stage of the disease, a subtle effect exerted by soluble forms of Aβ and tau at the synapse seems to be the earlier event underlying memory loss [98, 99, 107, 108]. Several studies have demonstrated that administration of different preparations of oligomeric Aβ and tau (synthetic, from transgenic mice, from AD brains) impaired synaptic plasticity and memory. The role of soluble oligomers also emerged in studies performed on AD mouse models, since synaptic and memory dysfunction was present before the appearance of plaques or tangles [18, 109].

In vitro and *in vivo* studies have shown that Aβ and tau derange molecular signaling pathways crucial for synaptic plasticity at both pre- and post-synaptic sites. Both Aβ and tau interfere with the transcription factor cAMP response element-binding protein (CREB), whose phosphorylation at Ser133 is thought to be one of the fundamental events in memory formation [110–112]. In particular, Aβ inhibits the physiological increase of CREB phosphorylation during LTP [113–115], causing a downregulation of both the NO/cGMP/PKG and the cAMP/PKA pathways, two cascades converging on CREB. Tau overexpression and hyperphosphorylation was also found to be accompanied by a reduction of CREB phosphorylation at Ser133, mediated by a decrease of phosphorylation of NR2B (Tyr1472) [116]. Moreover, synaptic plasticity and memory impairment caused by h-tau overexpression was reported to be related to nuclear dephosphorylation/inactivation of CREB [117]. Interestingly, these findings were validated in humans affected by AD showing a decrease in CREB and phospho-CREB levels in hippocampus [118–122].

Aβ and tau also target other molecules upstream of CREB, among which the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), another key molecule needed for LTP and memory formation [123]. CaMKII is dysregulated in the hippocampus of AD mouse models and patients (for a review, see [124]) and it has been demonstrated that Aβ oligomers interfere with its phosphorylation leading to AMPA receptor dysfunction [125–127]. On the other hand, evidences for the interaction tau-CaMKII have been reported since the late 1980 s in works analyzing the ability of CaMKII to induce an AD-like tau phosphorylation [128, 129]. CaMKII phosphorylates tau at different sites and this might prevent tau binding to microtubule [130] and modify tau structure leading to PHFs formation [131]. Indeed, CaMKII colocalizes with tau mRNA, PHFs, NFTs in AD brains (for

a review, see [124]). Recently, in a drosophila model of tauopathy, suppression of tau phosphorylation at Ser262/356 inhibited tau toxicity through a mechanism involving calcium homeostasis dysregulation driven by CaMKII [132].

The deleterious effects of Aβ and tau also involved BDNF, a critical factor linked to neuronal survival and function that is needed for synaptic plasticity and memory. A decrease of BDNF levels in serum and brains of AD patients correlates with cognitive impairment, and BDNF polymorphisms have been proposed to be involved in AD pathogenesis [133]. Moreover, several *in vitro* and *in vivo* studies have confirmed that Aβ-induced LTP and cognitive dysfunction are associated with a reduction of BDNF levels [133]. Recently, a loss of BDNF has been also reported in THY-Tau22 and P301L mouse models of tau pathology [134, 135].

Taken all together, these findings suggest that restoring synaptic-related molecules and second messenger systems regulating memory mechanisms might be a viable therapeutic strategy to counteract AD [115]. Most importantly, these data point at common synapse-related mechanisms affected by both Aβ and tau during memory impairment.

Aβ AND TAU ACTIVITY-DEPENDENT SECRETION, NEURONAL UPTAKE, AND SPREADING OF THE DISEASE

Because Aβ and tau interfere with the synaptic machinery, another relevant subject of investigation has been to determine whether they act via extracellular or intracellular mechanisms. Based on the localization of insoluble deposits, for several years Aβ has been considered an extracellular protein and tau an intracellular one. However, it is now clear that this rigid vision is no more applicable, since both Aβ and tau can be found inside and outside neurons. Notwithstanding most of the studies have been performed on models of disease, the extra- and intracellular presence of Aβ and tau is the result of a physiological dynamic process in which the two proteins are secreted at the synapse and internalized by neurons. A relevant body of data has supported the hypothesis that neurons are able to secrete Aβ in an activity dependent fashion. *In vitro* studies performed by applying drugs that decrease (i.e., tetrodotoxin or high magnesium) or increase (i.e., picrotoxin) neuronal activity have shown a concomitant decrease or increase of Aβ secretion in organotypic slices overexpressing human AβPP Swedish mutation [136]. An *in vivo* approach by using microdialysis also revealed an increase of Aβ levels in the brain interstitial fluid concomitant to the increase of synaptic activity [137] or paralleling the neurological status [138]. An increase of Aβ secretion has also been found during learning in healthy wild-type mice [38]. Based on the fact that synaptic activity stimulates Aβ secretion, and that extracellular Aβ is known to reduce synaptic plasticity, it has been proposed a theory according to which an increase of synaptic (and cognitive) activity is linked to AD pathogenesis. However, although an increase of brain activity in AD could be supported by data indicating hyperexcitability in transgenic mice and human AD patients [139, 140], this activity-dependent role of Aβ should be better viewed as a physiological mechanism occurring within the healthy brain, especially because levels of Aβ secreted during activity are in the picomolar range and are not neurotoxic [35, 38, 141]. Thus, the high increase of extracellular Aβ during AD might be due to a derangement of this physiological loop or it could be a consequence of degeneration of neurons that have previously accumulated Aβ at intracellular level (for a review, see [142]). Whether the impairment of synaptic function is directly mediated by these high extracellular Aβ levels or by Aβ accumulated inside neurons, is still a matter of debate. Surely, these two pools are strictly interconnected, since extracellular Aβ induced the accumulation of intracellular Aβ by stimulating AβPP processing [143] or by a direct AβPP-mediated internalization [144]; in turn, intracellular Aβ disrupts synaptic transmission and plasticity [145].

Interestingly, tau also undergoes the same dynamic flux characterized by activity-dependent secretion and neuronal internalization. Indeed, application of KCl or glutamate to cultured neurons resulted in an increase of tau secretion [98, 146] mediated by AMPA receptor activation [146]. *In vivo* studies reported an increase of tau in brain interstitial fluid when stimulating neurons with high K^+ perfusion, or after stimulation of the *N*-Methyl-d-aspartic acid (NMDA) receptors, or picrotoxin administration [147]. An increase of tau secretion also paralleled the increase of glutamate release induced by an antagonist of metabotropic glutamate receptors 2/3 [147]. The phenomenon was further confirmed in different cultured neural cell lines where extracellular tau levels were modified proportionally to synaptic activity [148]. On the other hand, several pre-clinical studies have demonstrated that exogenously applied tau can

be internalized by neurons [98, 149–152] and glial cells [153–155] with different mechanisms involving bulk endocytosis [152], binding to heparan sulfate proteoglycans [156] or to AβPP [144].

Activity dependent secretion and neuronal uptake of Aβ and tau have been related to the spread of the disease throughout the brain, a process known as spreading which refers to the capability of neurotoxic proteins to diffuse from a neuron to another, expanding the disease from a restricted area to the entire brain. This type of dissemination, defined as "trans-synaptic spreading", is thought to occur among different brain areas functionally connected [157, 158] and is supported by observations on postmortem AD brains as well as by clinical studies exploiting computerized x-ray tomography (CT) and magnetic resonance imaging (MRI) techniques, that allow tracing different neuropathological markers such as atrophy of certain brain areas, brain ventricles enlargement, and deposition of amyloid plaques and NFTs (for a review, see [78]). However, it should be pointed out that imaging biomarkers like fluorodeoxyglucose in PET scans are associated to discrete difficulties in data interpretation, as they are also positive in Suspected Non-Alzheimer Disease Pathophysiology (SNAP) [159].

Evidence for AD spreading and progression throughout the cortex was reported more than 30 years ago, based on tangle distribution in the proximity of the same pyramidal neurons that give connectivity to other brain areas [160]. At the present day, neither the cause that initiates spreading nor its underlying mechanisms have been identified, but useful information has come from pre-clinical studies. Notwithstanding tau has been under the spotlight for many years, one of the first evidence of spreading in AD dates back to the 1990 s and involves Aβ [161, 162]. When trying to unravel the causes of Aβ diffusion, studies have often focused on the first area affected in AD, the medial temporal lobe, and in particular, the entorhinal cortex (EC). EC superficial layer is susceptible to Aβ-dependent neurodegeneration, and this can negatively affect its primary afferent regions resulting in a disruption of the whole circuitry in both mouse models and AD patients [163, 164]. Consistently, an increase of mutant AβPP in layer II/III neurons of EC has been shown to elicit a molecular and functional disruption in the CA1 area of the hippocampus with presence of soluble Aβ in the dentate gyrus, Aβ deposits in the performant pathway, and epileptiform activity in the parietal cortex [165]. Further studies in mutant human AβPP (mhAβPP) mice have reported an age-dependent progressive deterioration of synaptic plasticity and memory spreading from the EC to the hippocampus [166], a phenomenon mediated by microglial RAGE activation and subsequent increase in p38MAPK phosphorylation [166]. Consistently, other studies reported the capability of reactive microglia in secreting Aβ through microvesicles, which in turn would promote Aβ toxicity to neurons through their axons [167–169]. Accordingly, other supporting evidences indicate that after administration of fluorescent oligomeric Aβ to neurons, a higher percentage of the protein was found surrounding neurons, and this process needed the presence of differentiated neuritis to occur [170]. Cell-to-cell transfer mechanism has been reported for different Aβ species (i.e., oAβ1–42 TMR, oAβ3(pE)–40TMR, oAβ1–40TMR, and oAβ11–42TMR), and this prion-like spreading was attributed to an insufficient activity of cellular clearance degradation systems [171]. Another mechanism proposed for Aβ spreading relies on the presence of tunneling nanotubes (TNTs) consisting of cellular membrane extensions creating a direct connection between cells [172]. TNTs have been demonstrated to mediate high-speed transfer of Aβ among neurons, through a p53/EGFR/Akt/PI3K/mTOR pathway that, in turn, would trigger F-actin polymerization promoting TNTs formation [173]. However, Aβ has been shown to be secreted by neurons through exosomes [174] that could be internalized and stored from the acceptor neuron as lysosomal vesicles through a macroautophagy mediated mechanism [170, 175]. In any case, despite these numerous evidences, there is not a uniform consensus about the causes or mechanisms underlying Aβ spreading.

On the other hand, a growing body of evidence refers to tau spreading as a prion-like propagation, which fascinatingly occurs in different directions among the many forms of tauopathies [176]. Also, tau pathology is likely to begin in EC then move to the hippocampus, and ultimately invading the cortex, following an overlapping path existing among functionally connected areas [55, 157, 158, 177]. These evidences are consistent with data coming from studies on non-human primates in which bilateral lesions of EC induce a functional impairment of declarative memory accompanied by long-lasting hypometabolism in temporal and parietal lobes, demonstrating a functional connection starting from EC [178]. Accordingly, in a transgenic mouse model differentially expressing pathological human tau in

EC (EC-tau), the localization of tauopathy was investigated at different time points, demonstrating a progression of the pathology through anatomically and functionally connected brain areas [158]. Interestingly, *in vivo* chemogenetic stimulation of EC in EC-tau mice induced additional pathology in synaptically connected areas (e.g., dentate gyrus) [148]. Consistent with this finding, tau has been found in exosomes that might lead to its diffusion to adjacent cells [106, 179]. Further work demonstrated that cell-to-cell contact was not necessarily needed for tau spreading *in vitro* given that the administration of neuronal-derived tau media to neuronal cultures was sufficient for tau transfer and internalization, even though it is not known whether tau in the media was vesicle bound or free [148]. Other studies suggested that pathologic tau requires TNTs to be transferred from a neuron to another one [180]. However, whether the mechanism underlying tau propagation is mediated by TNTs, non-vesicular direct translocation or through secretory lysosomes into extracellular space [106, 162, 181, 182] is still under investigation. Another interesting feature of tau transmission is the possibility that it can move both anterogradely and retrogradely, meaning that it can be internalized both at the somatodendritic compartment and axon terminals, and can be transported in either direction to disseminate tauopathy [152, 162].

While spreading is involved in the progression of the disease among functionally connected brain areas, the transition from oligomers to insoluble deposits has been described as a "nucleation-dependent protein polymerization" and explains the pattern of aggregate formation [183] for proteins with high tendency to organize in β-sheet conformation as for Aβ, tau, or α-synuclein [184]. This process, known as seeding, involves a nucleation phase and a growth phase. In the nucleation phase, the nucleus formation requires the assembly of misfolded monomers, a thermodynamically unfavorable process remarkably dependent on protein concentration [161, 185, 186]. The latter influences the lag time defined as the period before aggregates detection. In fact, supersaturated solutions can drastically shorten the nucleus formation time from years to microseconds [161]. After the nucleus formation, the critical concentration is reached, and a further addition of monomers occurs leading to polymerization, representing the growth phase. Interestingly, if a preformed nucleus, or *seed*, is added to a solution containing normally folded monomers, an immediate polymerization occurs. This phenomenon is defined as *seeding* [161, 183] and can be distinguished as homologous or heterologous [183, 187]. While homologous seeding involves monomers of the same type, heterologous seeding or cross-seeding takes place when a nucleus formed by a certain misfolded protein promotes polymerization of a different protein [183, 187]. A large body of evidence supports this cross-seeding among tau, α-synuclein and TDP-43 [188]. Some studies in which spreading of tau pathology was significantly accelerated by injecting pre-aggregated Aβ into mouse brain [189, 190] suggested the possibility of Aβ and tau cross-seeding. Consistently, a protein interaction study by surface plasmon resonance demonstrated an affinity constant of tau for Aβ which was almost 1000-fold higher than for tau toward itself [191]. Moreover, confocal immunohistochemical imaging of AD brains showed intracellular aggregates in which Aβ and tau coexisted in the same structure [191]. Also, a recent work showed that tau fibrillization can be induced in a cell-free assay by adding pre-aggregated Aβ, and that Aβ provide an efficient seed to induce tau cross-seeding and a consequent spreading of tau pathology *in vivo* [192].

In conclusion, seeding and spreading of Aβ and tau and their dynamic flux across the membrane characterized by activity-dependent secretion and neuronal internalization are crucial for the progression of the disease. Most importantly, the commonalities displayed by both Aβ and tau with respect to these phenomena are intriguing and suggest that soluble forms of the two molecules are involved in similar mechanisms of disease etiopathogenesis.

ALZHEIMER'S DISEASE: REARRANGING THE PUZZLE

As described above, Aβ and tau share several features leading to common mechanisms of toxicity, regardless of their different sequence (Table 2). This was predicted by a study showing that all of the soluble oligomers tested including α-synuclein, islet amyloid polypeptide, polyglutamine, lysozyme, human insulin, and prion peptide 106–126, display a common conformation-dependent structure that is unique to soluble oligomers [193]. By now, a variety of studies have demonstrated that soluble oligomeric forms of Aβ and tau, more than their aggregates, are increased in the diseased brain, are detectable in the CSF, and are highly correlated with cognitive impairment. The deleterious effect of Aβ and tau occurs

Table 2
Similarities and differences between Aβ and tau

	Amyloid-β Peptide	Tau Protein
Isoforms	• $A\beta_{40}$, $A\beta_{42}$, other fragments	• 3R-4R, 0N-1N-2N
Secondary structure	• β-sheet	• β-sheet
Physiological functions	• Neuronal growth • Neurotransmitter release • Synaptic transmission and plasticity • Memory formation • Immune response • Anti-oxidant properties	• Microtubule assembly and stabilization • Axon elongation • Synaptic plasticity Nuclear function
Aggregation sequence	Monomers → Oligomers → Fibrils → Senile plaques	Tau hyperphosphorylation → PHFs → NFTs
Insoluble and soluble forms	• No correlation between senile plaques and cognitive impairment • Oligomers induce synaptic dysfunction and memory loss • Oligomers increase in brains and CSF of AD patients versus controls	• Poor correlation between NFTs and cognitive impairment • Oligomers induce synaptic dysfunction and memory loss • Oligomers increase in brains and CSF of AD patients versus controls
Genetic mutations	AβPP, PS1 and PS2 linked to FAD	MAPT linked to FTDP-17, PSP, CBD
Synaptic target	CREB, CamKII, BDNF among others	CREB, CamKII, BDNF among others
Extra- and intracellular dynamic	• Activity dependent secretion • Neuronal and glia uptake • Extracellular toxicity • Intracellular toxicity	• Activity dependent secretion • Neuronal and glia uptake • Extracellular toxicity • Intracellular toxicity
Spreading	EC → Hippocampus → Cortex	EC → Hippocampus → Cortex
AβPP-dependent mechanism	• AβPP binding • Neuronal and glial uptake • Synaptic plasticity impairment • Memory impairment	• AβPP binding • Neuronal and glial uptake • Synaptic plasticity impairment • Memory impairment

PHFs, paired helical filaments; NFTs, neurofibrillary tangles; CSF, cerebrospinal fluid; AβPP, amyloid-β protein precursor; PS, presenilin; FAD, familiar Alzheimer's disease; MAPT, microtubule-associated protein tau; FTPD-17, frontotemporal dementia with parkinsonism-17; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; CREB, cAMP response element binding protein; CaMKII, Ca2+/calmodulin-dependent protein kinase II; BDNF, brain-derived neurotrophic factor; EC, entorhinal cortex.

at the synapse, where they interfere with molecular pathways needed for synaptic plasticity and memory. The capability of neuronal and glial cells to release and internalize Aβ and tau contributes to spread of the disease from specific areas, such as EC and the hippocampus, to the entire brain. Despite these studies have certainly clarified several aspects of AD onset and progression, the crosstalk between Aβ and tau in the diseased brain is still a matter of debate.

The most common view in the AD field is based on the "Amyloid Cascade Hypothesis" and suggests that the initial increase of Aβ induces amyloid and tau pathology over time (Fig. 2). This temporal sequence derives from studies in patients with FAD, where the genetic-driven increase of Aβ is followed by NFT accumulation [194], whereas the increase of tau, as in tauopathies, is not associated with Aβ deposition. Preclinical studies have confirmed that oligomers of Aβ can trigger tau pathology [195] and, conversely, when knocking down tau, Aβ toxic effects are prevented [196, 197]. Interestingly, recent work has demonstrated that Aβ acutely induces tubulin post-translational modifications and stabilizes dynamic microtubules promoting tau-dependent loss of dendritic spines and tau hyperphosphorylation [52]. Thus, Aβ has been thought to act upstream of tau in the pathogenesis of the disease. However, our recent works have challenged this scenario. We have demonstrated that oligomers of both Aβ and tau produce an immediate reduction of synaptic plasticity and memory when extracellularly applied and that these detrimental effects occur not only with high concentrations of Aβ or tau alone, but also when sub-toxic doses of oligomeric Aβ are combined with sub-toxic doses of oligomeric tau [98]. These observations suggested that: 1) Aβ and tau might act at the same level or on different targets that later converge on a common molecular mechanism; 2) the two proteins are able to impair synaptic plasticity and memory *per se*, i.e., regardless of the presence of high concentrations of one another; and 3) elevated levels of Aβ are not needed to initiate the tau-mediated toxic events leading to synaptic dysfunction. Inspired by these data, we have recently focused on the possible common mechanism of action for extracellular Aβ and tau oligomers to impair LTP and memory.

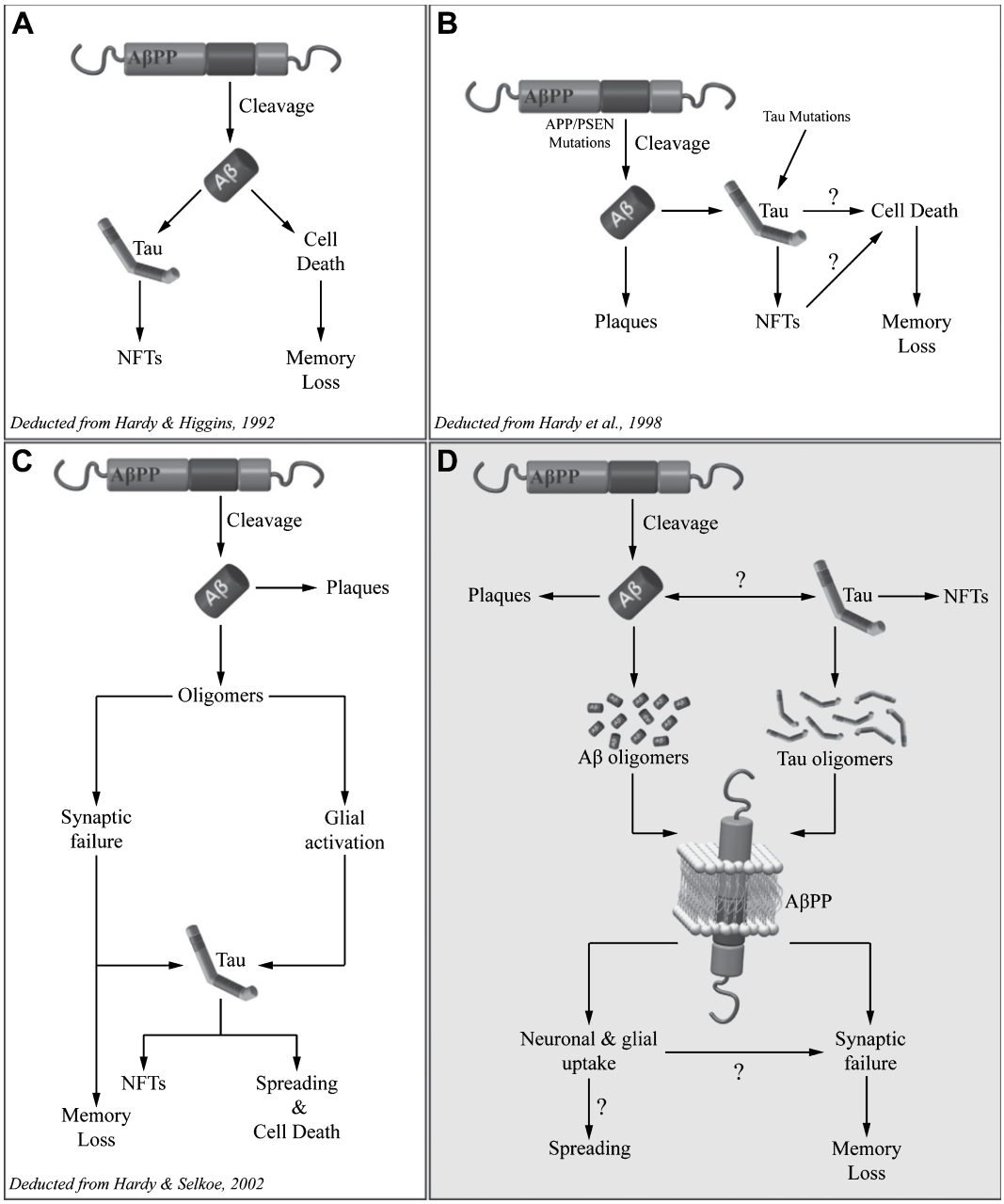

Fig. 2. Different views of Aβ and tau interaction in AD pathogenesis. The Amyloid Cascade Hypothesis has dominated the AD field for several years. This picture describes how it has been updated over time from the beginning (A), to the discovery of genetic mutations involving both Aβ and tau production (B), to a more complex vision recognizing oligomers as the toxic Aβ species (C). Notably, in A-C Aβ acts upstream tau. D) According to our novel vision, both oligomers of Aβ and tau exert a neurotoxic effect mediated by AβPP leading to synaptic and memory dysfunction. AβPP also mediates oligomers entrance into neurons and glial cells, a mechanism probably contributing to the spreading of the disease throughout the brain.

We found that both oligomers of Aβ and tau require AβPP to exert their deleterious effect at the synapse [144], in agreement with previous observations indicating that AβPP mediates extracellular Aβ neurotoxicity [143, 198, 199], and a recent study showing that AβPP is required for binding of human brain-derived oligomers to synapses and disruption of synaptic plasticity [200]. Our findings are also consistent with the observation that AβPP is involved in AD hippocampal hyperactivity [140, 201–204].

Previous papers have already shown that oligomeric Aβ is able to bind AβPP [205], whereas AβPP and tau interaction was studied several years ago in the context of NFTs [206–208]. We have now provided evidence that soluble oligomeric tau is able to bind AβPP [144]. This binding might be related to the AβPP-mediated uptake of Aβ and tau, since AβPP influences accumulation of tau fibrils in cultured cells [209] and is needed for the entrance of oligomeric Aβ and tau into neurons [144] and astrocytes [155]. Based on these findings, we hypothesize that AβPP-mediated oligomer uptake plays a role in AD pathogenesis. Indeed, because Aβ and tau do not impair synaptic plasticity and memory in AβPP KO mice, AβPP binding and/or AβPP-mediated internalization of the two proteins should lead to LTP and memory reduction, even if one cannot exclude the possibility that Aβ and tau act on other targets, or that their intraneuronal accumulation does not directly inhibit the synaptic machinery. However, a previous observation indicating that blocking intracellular Aβ rescues the LTP impairment induced by administration of extracellular Aβ [145] supports the hypothesis that Aβ intraneuronal uptake is critical for the impairment of synaptic plasticity. On the other hand, recent studies have evidenced that the AβPP-dependent accumulation of extracellular tau oligomers in astrocytes induces a disruption of calcium signaling which in turn disrupts synaptic function in neighboring neurons [155]. Interestingly, while it has been previously demonstrated that extracellular Aβ requires AβPP cleavage to permit intraneuronal Aβ accumulation [143], our results have excluded that the toxicity of extracellular Aβ and tau oligomers on LTP depends upon amyloidogenic processing of AβPP since BACE KO mice still present the impairment of LTP induced by the two oligomeric proteins [144].

The requirement for AβPP to lead to intracellular entrance of Aβ and tau oligomers to produce synaptic dysfunction and memory loss begs the question of how this occurs. Whether AβPP acts as a channel permeable to the oligomers [210, 211], or induces the formation of pores across the membrane to let oligomers enter the cell [212], or promotes endocytosis of the oligomers [213], is still under investigation. Another possibility is that when Aβ and tau oligomers bind AβPP, they lead to the activation of its intracellular portion, AID/AICD, triggering either a structural change, for example inducing a different AβPP conformation, or a functional effect, for example activating or inhibiting molecular cascades involved in synaptic plasticity and memory. Interestingly, it is known that AID/AICD might stimulate transcription by forming a multimeric complex with the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60 [214]. It has been also shown that AβPP-dependent transcription mediated by Fe65 is blocked by the cell death mediator p75, which is able to bind AβPP altering its processing [215]. Another possible mechanism might involve AβPP phosphorylation at specific intracellular sites. For example, it has been demonstrated that AβPP phosphorylation of Thr668, which regulates docking sites for intracellular proteins that interact with AβPP, is increased in AD cases [216] and knock-in mouse bearing a Thr(668)Ala mutation preventing phosphorylation at this site protects against abnormal synaptic plasticity and memory when crossed with a mouse model of dementia [217].

Our model placing extracellular Aβ and tau in parallel and upstream of AβPP does not exclude the possibility that the two proteins involve other molecules to produce detrimental effects in addition to synaptic plasticity and memory impairment, nor the possibility that some deleterious effects need the other protein for the effect itself to be present (i.e., Aβ might require tau for some of the pathologies to occur). Consistent with this scenario, AD is a complex condition involving multiple aspects in addition to memory, a phenomenon that is likely dependent upon synaptic activity and that has greatly influenced our critical analysis of the current literature because it represents the clinical hallmark of AD. Furthermore, as shown in Table 2, some of the physiological functions of the two proteins are different with Aβ playing a major role in neuronal growth and synaptic plasticity and tau in axonal elongation and microtubule assembly and stabilization. Then, in light of the different affinities that Aβ has towards its multiple targets, it is likely that as the concentration of the peptide increases with worsening of the pathology new pathways are affected by the disease.

In any case, demonstrating that AβPP serves as a Trojan horse to mediate synaptic plasticity and memory impairment by extracellular oligomers of both Aβ and tau, challenges the prevailing hypothesis in the AD field stating that Aβ triggers tau pathology. According to our findings, Aβ and tau do not act in series but in parallel, both through AβPP (Fig. 2). Now, it would be desirable to understand whether and how the involvement of AβPP is limited to Aβ and tau entrance into cells or also underlies the derangement of molecular mechanisms involved in synaptic

plasticity and memory. In any case, this new player might be taken into consideration when studying the pathogenesis of AD. For example, further studies should be performed to understand the exact mechanisms of AβPP-mediated entrance of oligomers inside neurons and glial cells and whether this might initially represent a compensatory mechanism aimed at clearing toxic oligomers from the synaptic cleft.

The consequences of the model underlying AD pathology proposed in the current review are notable from a drug discovery point of view. The first thought is that therapies targeting tau might not work similarly to the failure of anti-Aβ therapies, as Aβ might still exercise its detrimental effects independent of tau and *vice versa*. Most important, given the convergence of Aβ and tau onto AβPP, a fascinating possibility is that therapies acting onto AβPP might be more efficacious than those acting solely against Aβ or tau. Furthermore, an approach directed against AβPP would have the advantage of overcoming obstacles offered by the physiological functions of Aβ and tau that might occur independently of their action onto AβPP and might still be present if one decides to simultaneously target Aβ and tau, an approach that is also suggested by our model. A strategy directed against AβPP will likely have its own drawbacks including physiological functions of full length AβPP [4]. Nevertheless, AβPP offers the flexibility of having multiple sites undergoing post-translational modifications that could be exploited as a tool to selectively affect a putative AβPP-dependent toxicity of Aβ and tau oligomers [218]. To this end, the AβPP phosphorylation at Thr668 is very interesting because it has been suggested that averting its noxious role in synaptic plasticity and memory might serve as a therapeutic strategy for human dementias [217]. Consistent with this finding it has been shown that overexpression of the protein phosphatase 2A (PP2A) methyltransferase, leucine carboxyl methyltransferase-1, leads to a decrease in AβPP phosphorylation at the PP2A-sensitive Thr-668 site and protects mice against Aβ-induced damage of synaptic plasticity and memory [219]. Certainly, our hypothesis paves the way to an increased interest toward AβPP, a molecule that has been taken into account mostly for its role as an Aβ generator, being, in our opinion, unfortunately overshadowed by its own child, Aβ.

In conclusion, after more than one century of research in the AD field, several questions remain to be answered especially on the role of the two main actors, Aβ and tau, in the pathogenesis of the disease. It is certain that their interactions at the synapse need to be further elucidated and new players such as AβPP should enter the stage to get a clearer picture of this intricate disease.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9935).

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Amyloid Accumulation and Cognitive Decline in Clinically Normal Older Individuals: Implications for Aging and Early Alzheimer's Disease

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Abstract. The aberrant accumulation of the amyloid protein is a critical and early event in the Alzheimer's disease (AD) cascade. Given the early involvement of this pathological process, it is not surprising that many clinically normal (CN) older individuals demonstrate evidence of abnormal Aβ at postmortem examination and *in vivo* using either CSF or PET imaging. Converging evidence across multiple research groups suggests that the presence of abnormal Aβ among CN individuals is associated with elevated risk of future clinical impairment and cognitive decline. Amyloid positivity in conjunction with biomarkers of neuronal injury offers further insight into which CN are most at risk for short-term decline. Although in its infancy, tau PET has demonstrated early increases among Aβ+ that will likely be an important indicator of risk among CN. Overall, the detection of early Aβ among CN individuals has provided an important opportunity to understand the contributions of this pathology to age-related cognitive decline and to explore early intervention with disease modifying strategies.

Keywords: Aging, amyloid, biomarkers, cognitive decline, early detection, memory, PET

BACKGROUND: ALZHEIMER'S DISEASE AND AMYLOID

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that typically begins with episodic memory impairment and eventually impairs the ability to function independently. Over 5.5 million Americans are currently living with AD dementia, with approximately 10% of individuals over age 65 and 40% of individuals over age 85 impacted. There are no disease-modifying treatments that directly target the underlying disease. Current treatment options offer only mild relief of symptoms.

AD dementia is characterized pathological by the presence of two hallmark protein aggregations—amyloid-β (Aβ) into plaques and phosphorylated tau into neurofibrillary tangles (NFTs) [1, 2]. Whereas the aberrant accumulation of the amyloid protein is considered an early initiating event in the AD cascade [3], the spread of tau within the medial temporal lobe and into neocortex is thought to occur downstream to abnormal accumulation of amyloid

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and is more proximal to clinical symptoms of dementia [4–6]. However, the exact sequence of events involving these two hallmark pathological features of the disease, as well as the mechanisms by which these pathological aggregations influence neuronal integrity and clinical symptoms is unknown, and is currently under investigation in biomarker studies that aim to visualize and track these pathologies throughout the course of AD [7].

Although multiple biomarkers exist to capture different aspects that occur throughout the AD continuum, the focus of this review will be primarily on the measurement of Aβ via positron emission tomography (PET) imaging, and how this technology has been applied to clinically normal cohorts to identify individuals with evidence of early AD pathology. ^{11}C-PIB ('Pittsburgh Compound-B') was one of the first radiotracers to enable the visualization of Aβ plaques [8], with imaging-postmortem studies showing high correspondence between *in vivo* signal and moderate to frequent amyloid plaque pathology at autopsy [9, 10]. The success of ^{11}C-PIB in the research setting accelerated the development of ^{18}F amyloid PET compounds that have greater feasibility given the shorter half of ^{18}F isotopes (110 minutes for ^{18}F compared to 20 minutes for ^{11}C). The longer half-life enables ^{18}F compounds to be delivered over a long distance from distribution centers rather than depending on an on-site cyclotron; thus, the overall utility of amyloid PET in research and in clinical settings has dramatically increased over recent years. Between 2012 and 2014 there were three ^{18}F compounds approved by the FDA to assess Aβ deposition in patients with clinical symptoms—florbetapir/Amyvid [11], flutemetamol/Vizamyl [12], and florbetaben/Neuroceq [13]. Thus, in addition to the large role of amyloid PET imaging in specialized research settings, it has become increasingly common across medical and research settings across the globe. Given the lack of disease modifying treatments for AD dementia, the utility of amyloid PET in the clinical setting is a topic of debate and large-scale studies are currently underway to understand the impact of amyloid PET in the clinical setting according to "appropriate use criteria" [14], specifically, in impaired patients that are suspected to have AD dementia but have atypical non-amnestic clinical presentations, as well as in mild cognitive impairment (MCI) patients, a population that is heterogeneous with various contributing etiologies. This multi-site study of over 18,000 Medicare beneficiaries, the Imaging Dementia-Evidence for Amyloid Scanning (IDEAS), will provide important insights into how amyloid PET scans influence patient management and medical outcomes [15].

PREVALENCE OF Aβ IN CLINICALLY NORMAL OLDER INDIVIDUALS

Although Aβ plaques are a central feature of AD dementia, they are also commonly observed in the brains of clinically normal (CN) older individuals that do not show signs of objective cognitive impairment as detected with neuropsychological assessment. This observation has consistently been observed in postmortem studies [6, 16], cerebrospinal fluid (CSF) studies [17, 18], and amyloid PET imaging studies [19]. The prevalence of CNs with evidence of elevated Aβ (Aβ+) increases with older age as well as the APOE4 genotype [20, 21], with little evidence of abnormal Aβ accumulation before age 60. Interestingly, the regional distribution of amyloid plaques throughout the brain tends to be widely distributed with involvement across multiple association cortices [22]. This global distribution pattern is common among Aβ+ CN, suggesting that specific focal regions do not seem to be susceptible to amyloid accumulation among older individuals (at least using the current amyloid PET ligands). However, some work has suggested that large areas encapsulating highly connected heteromodal cortical regions seem to be most impacted by Aβ deposition [23, 24].

The presence of abnormal Aβ accumulation within CN individuals is consistent with models of AD suggesting that Aβ is an early initiating event that eventually leads to "downstream" brain changes and clinical impairment [3, 7]. Consistent with this framework, Aβ+ CN individuals are at greater risk of gray matter atrophy in the medial temporal lobe as well as lateral association cortex when assessed longitudinally [25], which may reflect downstream changes Aβ-related toxicity. Interestingly, longitudinal studies examining the rate of Aβ accumulation over time among CN has shown very slow rates, suggesting that this process may occur for decades before neurodegeneration is clearly evident and clinical symptoms of dementia are present. Specifically, Villemagne and colleagues have estimated that it may take 20 years to transition between Aβ levels typically found in Aβ+ CNs compared with Aβ levels found in AD dementia, highlighting the prolonged period over which Aβ accumulation may occur within CN individu-

als before clinical symptoms of dementia are present [26]. Given this prolonged stage of abnormal Aβ accumulation that occurs during among CN individuals, much research has focused on identifying subtle changes that occur in brain structure and function during this stage. These studies have suggested that Aβ+ CN show subtle decreases in gray matter measures [27, 28], resting state connectivity [29–31], as well as task related activation during memory processing [32, 33], highlighting that early effects of this pathology on brain structure and function can be detected concurrently with abnormal levels of Aβ, before clinical symptoms of dementia.

COGNITIVE DECLINE IN "NORMAL" AGING

Lifespan studies report age-related decrements in performance across multiple cognitive domains, including memory, working memory/executive functions, and processing speed in CN cohorts [34–36]. As an illustration, normative data suggest that recall of 8 words on the 15-word Rey Auditory Verbal Learning List (RAVLT) is normal performance for a 70-year-old woman whereas recall of 8 words would reflect borderline impaired performance in a 30-year-old woman. The largest age-related cognitive effects are observed in the domains of episodic memory and processing speed [37]. However, age-related decline at the group level is generally small with some estimates of annual decline ranging from between 2–4% of one standard deviation in individuals aged 50 + [36]. Although subtle multi-domain cognitive decline is generally associated with age, some aspects of cognition remain relatively stable including speech and language processing [38] and procedural memory [39]; in addition, there is evidence that vocabulary knowledge [37, 40] and other aspects of semantic memory [41] not only remain stable but may improve throughout the lifespan.

Methodological challenges to quantifying "normal" aging exist. Cross-sectional studies may suffer from covariance between age and sampling bias with 30-year-old and 70-year-old subjects reflecting fundamentally different cohorts with unique reasons for participating in research. Longitudinal studies must account for practice effects and selective attrition. For example, Josefsson et al. showed that age-related declines in memory over a 15-year longitudinal period were under-estimated prior to statistically accounting for attrition; participants who dropped out of the study were more likely to exhibit decline in their memory performance prior to study discontinuation [42].

Despite these methodological hurdles, multiple cross-sectional studies of cognitive aging show linear relationships between age and cognitive decline starting as early as in the late 20s. Park et al. has reported decrements in cognitive performance that were present linearly across the lifespan, suggesting that subtle cognitive decline occurs well before the ages in which risk of dementia is highest [43]. Other studies also suggest linear decline but with the addition of an inflection point around age 60–65 with a subsequently greater magnitude of age-related cognitive decrements [37].

The presence of this inflection point highlights both the theoretical and methodological challenge of differentiating benign versus pathological cognitive aging. More specifically, multiple risk factors for dementia (such as hypertension, diabetes) are both associated with age and, furthermore, may confer independent risk of normative cognitive decline. In addition, there is significant overlap between the most prevalent cognitive complaints in typical aging such as difficulty with proper name recall and weaknesses in memory retrieval which mirror the earliest cognitive signs associated with AD [44]. Given that age is the primary risk factor for AD dementia, the ability to measure AD pathology *in vivo* provides a unique opportunity to understand the contributions of early pathology to decline observed in aging, as well as potential interactions between "normal" and "pathological" aging.

ELEVATED RISK OF CLINICAL PROGRESSION AMONG Aβ+ CN

Studies that have examined older CN individuals in conjunction with Aβ status have consistently revealed that Aβ+ CNs have greater risk of progression on functional measures, such as on the clinical dementia rating scale [45] and progression to MCI and dementia [46]. Examining a mean follow up of 3.70 years (ranging between 1 and 7.5 years of follow up across participants), Roe et al. reported a hazards ratio of 3.68 describing risk of progression in Aβ+ versus Aβ– CN individuals classified according to PIB PET (with similar hazards ratios when CN were classified according to CSF amyloid levels rather than PIB PET) [45]. Likewise, a separate study from AIBL

of CN individuals reported odds-ratios of 4.8 when examining the proportion of Aβ+ CN that progressed to a clinical diagnosis of MCI or AD dementia after 3 years of follow up compared to Aβ– CN [46].

Although these aforementioned studies highlight that the Aβ+ CN group is at greater risk of clinical progression, it is important to note that the overall rates of progression are low for studies with short follow up durations (<4 years of follow up). Specifically, the aforementioned AIBL study by Rowe et al. reported 26% of Aβ+ CN progressed to MCI/dementia compared to 7% in the Aβ– group after 3 years. Recent work from Donohue and colleagues from the ADNI suggests although significant albeit low rates of progression on the Clinical Dementia Rating (CDR) scale are greater in Aβ+ CN compared to Aβ– CN 3 to 4 years after baseline, much larger rates of CDR progression are apparent after 6 years of follow up in the Aβ+ group [47], highlighting the slow time course in which clinically meaningful changes occur in CN cohorts.

GREATER LONGITUDINAL COGNITIVE DECLINE IN Aβ+

Observational studies investigating longitudinal decline using neuropsychological measures have converged to show that the Aβ+ CN group shows worse cognitive performance over time compared to Aβ– CN. Although some groups have identified specific decline in episodic memory among Aβ+ CN [48, 49], others have reported decline across multiple cognitive domains, such as executive function, semantic memory, and processing speed (see meta-analysis by Baker and colleagues [50]). Interestingly, we have found early changes in semantic fluency among Aβ+ CN that remains significant after controlling for non-semantic aspects of verbal fluency (i.e., phonemic fluency) [51]. Petersen and colleagues have published the largest study to date that examined prospective cognitive decline among CN classified at baseline as Aβ+ or Aβ– using PIB PET across 564 CN followed on average for 2.7 years [52]. This study also identified multi-domain cognitive decline, with significant differences between Aβ+ and Aβ– groups of –0.09 z-score units per year for a composite measure of executive function (Trail Making Test Part B and Digit Symbol Substitution) and –0.07 z-score units per year for a composite measure of memory (delayed recall measures from the Wechsler Memory Scale–Revised Logical Memory II delayed recall, Wechsler Memory Scale–Revised Visual Reproductions II, and the Auditory Verbal Learning Test).

Data driven approaches examining patterns of retrospective decline preceding dementia diagnosis have similarly suggested that measurement of decline across multiple cognitive domains is optimal for capturing the gradual decline that occurs prior to dementia onset [53, 54]. Given that decline may not be restricted to episodic memory changes during the preclinical stage, cognitive composites scores spanning multiple domains have been utilized to explore Aβ related decline in observational cohorts [49, 52, 55, 56] as well as integrated into cognitive endpoints in clinical trials targeting at risk CN [57]. In addition to showing significant cognitive decline, Aβ+ CNs also show decline in measures of global cognitive function that are established proxies for clinically relevant change, such as in the Mini-Mental State Examination [58] and Alzheimer's Disease Assessment Scale cognitive subscale [59, 60]. Thus, at the group level, there is consistent evidence that Aβ+ CN show worse cognitive performance over time in memory and also non-memory domains compared to Aβ– CN, as well as decline in global cognitive measures that are likely more proximal to clinically meaningful change.

IMPLICATIONS OF MULTI-DOMAIN COGNITIVE DECLINE

The presence of multi-domain cognitive decline among Aβ+ CN may reflect sequential involvement across different cognitive domains, such that impairments in episodic memory precede decline in executive function, and that these declines occur among CN prior to clinical impairment [61]. This is consistent with the notion that in typical presentations of AD, the most early and prominent cognitive feature is episodic memory loss which coincides with tau proliferation in the medial temporal lobe [62]. Another possibility is that there is heterogeneity in the patterns of cognitive decline among Aβ+ individuals, such that some Aβ+ CN show memory decline whereas others show decline in non-memory domains such as executive function or language. The notion of heterogeneity in clinical presentations of AD dementia has long been established, with clinically "atypical" presentations involving disproportionate deficits in executive function (behavioral/dysexecutive-variant AD), language (Logopenic progressive aphasia), and visuospatial processing (posterior cortical atrophy).

While these variants are infrequent, there is evidence for amnestic versus non-amnestic subtypes within relatively typical AD dementia [63, 64]. Importantly, these non-amnestic subtypes may be associated with distinct patterns of atrophy and NFT burden [65–67], as well as a younger age and the absence of the APOE4 risk allele. Interestingly, although patterns of atrophy and tau accumulation tend to correspond well with the clinical phenotype, amyloid is globally distributed in these different subtypes. Thus, there may be "vulnerable" brain networks for a given individual that influences the clinical presentation of AD that are not driven by the regional impact of Aβ plaques.

The role of disease heterogeneity in cognitive trajectories during the preclinical stage of AD has largely been understudied and may explain the presence of subtle declines in cognition that are not restricted to episodic memory among Aβ+ CN. As is the case when interpreting heterogeneous clinical symptoms among dementia patients, heterogeneity in cognitive decline among Aβ+ CN may reflect individual differences in response to late life amyloid rather than the regional distribution of amyloid itself, given that patterns of amyloid uptake tend to be widely distributed throughout cortex even among CN. For instance, differences in development, lifestyle factors, genetics, and/or co-morbidities such as cerebrovascular disease, synucleinopathies, and transactive response DNA binding protein 43 kDa (TDP-43) may be important indicators that explain individual differences in patterns of decline among older Aβ+ CN.

GREATEST COGNITIVE DECLINE IN Aβ+ CN WITH EVIDENCE OF NEURODEGENERATION

Although the Aβ+ CN group consistently shows worse cognition over time when followed longitudinally, these changes are small in magnitude and above the magnitude of decline needed to be diagnosed with MCI (typically <0.10 z-score units per year difference across Aβ groups [52, 68]). Thus, biomarkers that may capture underlying neurodegenerative processes may improve the identification of Aβ+ CN most at risk for short term decline, with the idea that Aβ+ CN that additionally have evidence of neurodegeneration may indicate a later preclinical stage than Aβ+ CN without evidence of neurodegeneration [69]. Along these lines, in 2011 the National Institute on Aging–Alzheimer's Association work group proposed staging criteria for preclinical AD that incorporated markers of Aβ with markers of neurodegeneration (ND) to facilitate research focused on understanding the asymptomatic stage of AD and the identification of CN individuals most at risk for future decline. This initial NIA-AA framework classified individuals into Aβ+ and Aβ– groups based on either CSF or PET markers. This framework also incorporated markers of ND, which at that time included CSF tau/pTau, hippocampus volume measured with structural MRI, and hypometabolism in regions impaired in AD dementia. Unlike CSF and PET measures of amyloid which are highly correlated [18], markers of ND vary in their associations which makes this dimension of the NIA-AA 2011 research framework less straightforward to implement and interpret than classification along the Aβ dimension [70]. Thus, selection of ND marker will likely influence which participants are classified as ND+. Implementation of this criteria results in four groups: preclinical stage 0 is defined as Aβ– /ND–, stage 1 is defined as Aβ+/ND–, and stage 2 is defined as Aβ+/ND+. The fourth group, Aβ–/ND+ CN individuals, was initially not described in the NIA-AA 2011 research guidelines and subsequently labeled as "suspected non-AD pathophysiology" (SNAP) [71], with the implication that non-AD etiologies contributes to an AD-like pattern of ND in this group. Stage 3 was also proposed within the NIA-AA framework to encapsulate Aβ+/ND+ individuals that show subtle memory decline or cognitive complaints. However, given the complexities of defining this group, many studies have elected to keep all Aβ+/ND+ CN together in the Stage 2 group rather than further dividing Aβ+/ND+CN into Stage 2 and Stage 3.

Despite concerns regarding discrepancies across ND markers, studies examining the proportion of CN classified across the proposed stages have been remarkably consistent [71–76]. In general, preclinical stage 0 CN comprise anywhere from 40 to 60%, stage 1 is about 10–20%, stage 2 is 5–15%, and SNAP is around 25%. A major contributing factor to these proportions is cohort age, with younger cohorts showing more biomarker negative individuals (Stage 0) than Aβ+ individuals (Stages 1 and 2). Using this biomarker staging framework, investigators have examined longitudinal clinical progression to either MCI or AD dementia across preclinical stages defined at baseline, as well as change in cognition over time. Among the studies investigating clinical progression, most studies to date suggest elevated risk of clinical progression in Stage 2 CN

compared to other groups, with unclear risk in the Stage 1 and SNAP groups [76, 77]. An important consideration for studies examining clinical change in CN is the small number of progressors across the four biomarker defined groups. For instance, in the study by Knopman and colleagues, 127 CN were classified as Stage 0, 44 as Stage 1, 46 as Stage 2/Stage 3, and 69 as SNAP. However, after one year of follow up, only 6 Stage 0, 5 stage 1, 11 stage 2/3, and 7 SNAP progressed to either MCI or AD dementia [76, 77]. Future studies with longer follow up will be needed to clarify risk of clinically meaningful progression among different biomarker staging frameworks.

Given the slow progression rates within CN, a number of studies have investigated cognitive decline as a function of baseline preclinical staging using the NIA-AA framework. These studies consistently show the greatest decline among Stage 2 individuals compared to all other groups [72–74]. However, the presence of cognitive decline among Stage1 and SNAP is inconsistent. For instance, Soldan and colleagues examined longitudinal change in a global cognitive composite using data from the BIOCARD study. At baseline CN were an average of 57 years old and followed for 11 years. Classification into preclinical stages was based on baseline CSF measures for both Aβ and tau. This study found that a slope difference of –0.05 z-score units per year between Stage 2 and Stage 0, and no differences between the other three groups (Stage 0, Stage 1, SNAP) [74]. In a study by Burnham and colleagues using data from the AIBL, CN were an average of 73 years of age at baseline and followed for 6 years. Classification was performed using amyloid PET and hippocampus volume for ND. In this study there was a slope difference of –0.25 z-score units per year for memory between Stage 2 and Stage 0, and also a slope difference of –0.08 z-score for global cognitive decline between Stage 1 and Stage 0 (with no difference between Stage 0 and SNAP) [72]. Finally, our work in the Harvard Aging Brain Study examined CN with an average age of 74 at baseline and followed for 4 years. Classification was performed using PIB PET and both hippocampus volume in conjunction with patterns of hypometabolism for ND [78]. Consistent with the other studies, we reported significant decline in global cognition between Stage 2 and Stage 0 (–0.22 z-score units per year difference). However, we also found a group difference between SNAP and Stage 0 (–0.07 z-score units per year) and no difference between Stage 1 and Stage 0. In a follow-up paper, we did not find any difference in the pattern of decline across biomarker stages when examining different cognitive domains rather than global cognition (memory versus executive function) [73]. Direct comparison across these studies is difficult given that a number of parameters vary that may influence cognitive trajectories—specifically, age at baseline, follow up duration, and ND classification. Nevertheless, across all these studies preclinical Stage 2 was consistently shown to have the greatest cognitive decline. Cognitive decline among Stage 1 and SNAP remains unclear and may be more susceptible to cohort and analytical differences across studies.

INITIAL STUDIES WITH TAU PET IN CN

In addition to Aβ plaques, intracellular aggregations of the tau protein into NFTs are the other hallmark pathological feature of AD. Interestingly, the regional involvement and time course of NFTs throughout the lifespan follows a different pattern compared to Aβ plaques [79]. Specifically, NFTs begin in the transentorhinal cortex (Braak I/II); spread to other portions of entorhinal cortex as well as the CA1 subregion of the hippocampus and adjacent inferior temporal cortex (Braak III); and then finally are deposited in additional hippocampal subregions and cortical regions (Braak IV and higher) [80, 81].

When considering the postmortem literature, there are three consistent observations regarding the overall involvement of NFTs and Aβ plaques with respect to age and clinical status: 1) NFTs in early Braak regions are common in middle age and ubiquitous in older age (50% of 50 year olds and 90% of 70 years old have NFTs in entorhinal cortex), whereas abnormal Aβ (in a globally distributed pattern) is present later in the lifespan compared entorhinal cortex tau (10% of 60 year olds and 30% of 75 year olds) [79, 82]; 2) exacerbation of NFTs in entorhinal cortex and beyond entorhinal cortex into neocortex is coupled with accumulation of Aβ [4, 5]; and 3) widespread neocortical NFTs (≥Braak V) are associated with clinical dementia and are very uncommon among CN individuals [6]. Thus, it is expected that among older CN individuals, NFTs will be common albeit restricted to the entorhinal cortex, whereas variations within the MTL and involvement of regions beyond entorhinal cortex (i.e., hippocampus and inferior temporal cortex) are expected among older CN individuals that additionally have abnormal Aβ (although widespread neocortical involvement of tau beyond inferior temporal cortex is not expected among CN).

Recent advancements in tau PET imaging now enable the visualization of NFTs [83, 84], enabling multimodal imaging studies that investigate both hallmark pathologies of AD (Aβ and tau) during the preclinical stage of AD. Initial work applying tau PET using the ligand F18-AV1451 in CN have shown that Aβ+ CN have greater levels of tau compared to Aβ– CN, especially in medial temporal lobe and inferior temporal cortex [85, 86]. These patterns are consistent with pivotal postmortem work by Price and Morris that showed elevated medial temporal lobe tau in CN with moderate and frequent plaque counts compared to CN with little evidence of Aβ plaque pathology [5]. Interestingly, tau PET among CN has shown only moderate associations with markers of ND used to classify CN into preclinical stages using the 2011 NIA-AA framework. For instance, elevated tau in the medial temporal lobe and inferior temporal lobe has been shown to relate moderately to hippocampal volume measures only among Aβ+ CN and not Aβ– CN [73]. Furthermore, even associations between tau PET and CSF tau are not highly correlated when samples are restricted to CN [87, 88]. Specifically, a lack of significant correlation between regional tau measured with AV1451 and CSF total tau or phosphorylated tau has been reported across two independent cohorts of CN [87, 88]. Contrary to these studies, Chhatwal and colleagues found significant associations between tau from CSF and PET from some regions among CN. Specifically, CSF phosphorylated tau shared 31% of variance with entorhinal cortex tau and 53% of the variance with inferior temporal tau [89]. Given that CSF and PET measures of tau capture distinct forms of this pathological process (tau protein in the CSF versus intracellular neuronal inclusions), it is not surprising that these measures are not highly concordant and may show different levels of sensitivity among CN. Importantly, studies that combine CSF and PET within CN will be able to directly determine whether these measures of tau provide sequential information relevant to early AD, and/or provide unique information regarding future risk of cognitive decline and clinical impairment.

An initial study by Scholl and colleagues applying AV1451 to CN found that elevated tau in the medial temporal lobe was associated with worse memory both at the time of the tau scan as well as retrospectively [90]. Future work in larger samples will be necessary to examine the independent and synergistic effects between Aβ and tau on cognition, as well as the ability to predict prospective cognitive decline following the tau scan. Given the potentially complementary information gained by tau PET and tau from CSF within CN [88], it will be informative to understand whether these tau measurements independently contribute to cognitive decline among Aβ+ CN.

SYNERGISTIC EFFECTS BETWEEN Aβ AND GENETIC RISK FACTORS

A priori genetic factors, such as genotypes from Apolipoprotein E (APOE) and brain-derived neurotrophic factor (BDNF), have also been shown to interact with Aβ status to accelerate longitudinal cognitive decline among CN individuals. We have shown that Aβ+ CN individuals that are also APOE4 + show greater short term decline in global cognition as well as memory over a median follow up period of 1.5 years than other groups (APOE4-/Aβ–, APOE4 + /Aβ–, and APOE4-/Aβ+) [91]. Although the APOE4 genotype is known to influence AD risk through pathways related to abnormal Aβ accumulation [16, 92], this genotype also effects neuronal integrity through Aβ-independent mechanisms. For instance, APOE4 genotype has been shown to impact the response to neuronal injury, with the apoE4 protein being less effective than apoE3/2 proteins in responding to neuronal injury [93]. It is therefore possible that in addition to promoting Aβ accumulation, the APOE4 genotype also confers greater levels of neuronal toxicity in response to Aβ accumulation, ultimately making this Aβ+/APOE4 + group the most susceptible to short term cognitive decline than Aβ+ that are APOE4-. However, given the earlier age of Aβ accumulation among APOE4 + carriers [16], it is also possible that APOE4 + CN individuals have harbored abnormal levels of Aβ for a longer duration than their Aβ+/APOE4- counterparts and are therefore at a more advanced preclinical stage of the disease.

Similar to the increased risk of cognitive decline identified in APOE4 + /Aβ+ CNs, in a study of 165 CNs followed over 3 years, Lim et al. demonstrated that Aβ+ CNs that also have the val66met BDNF polymorphism show greater rates of cognitive decline [94]. Although this polymorphism is not associated with greater levels of Aβ accumulation, it results in decreased production of the BDNF protein and impairment of neuronal and synaptic growth [95]. Thus, the combination of abnormal Aβ in conjunction with the val66met BDNF polymorphism may influence an individual's ability to tolerate underlying

levels of Aβ and more susceptible to Aβ related toxicity. Interestingly, in a follow up study by the same authors, independent effects were identified for both APOE and BDNF genotypes, such that Aβ+ CN that additionally have both genetic risk factors show the most rapid memory decline [96]. The additive effect across these two genetic loci implies that there are synergistic effects between genetic risk and Aβ among CN, suggesting that consideration of genetic risk factors may provide important information regarding immediate cognitive decline among biomarker positive CN.

SENSITIVITY AND SPECIFICITY OF COGNITIVE TESTING IN CN

Most neuropsychological measures were designed for the detection of impairment within clinical populations. These measures may thus be insufficient in their level of difficulty and degree of specificity among CN adults to reliably detect 1) subtle relationships between cognition and AD biomarkers and 2) AD biomarker-related cognitive decline, particularly at shorter follow-up intervals and at the earliest stage of preclinical AD. For example, the Face Name Associative Memory Exam (FNAME) originated from the cognitive neuroscience literature and involves learning and remembering names associated with faces. The task is not only challenging but may also have greater ecological validity as older adults commonly report difficulty with proper name recall. Worse FNAME performance has been associated with greater amyloid burden in CN adults [97]. The Memory Binding Test [98] has similarly been shown to be correlated with amyloidosis in CN adults [99]. This measure, along the lines of the Free and Cued Selective Reminding Test enhances learning and recall through use of a semantic association paradigm. Decrements in recall on these measures, particularly those that persist despite semantic cueing, are prototypical in MCI due to AD [100]. We recently found that although decrements in cued recall on the Free and Cued Selective Reminding Test were rare among CN adults, Aβ+ individuals were 3.55 times more likely to show cued recall decline and that decline was associated with greater risk of clinical progression on the clinical dementia rating scale [101].

Other cognitive measures with promise include the Short-Term Memory Binding Test which requires a participant to identify whether there has been a change in either the shape alone or shape and colors of polygons across trials. Feature binding in short-term memory has been associated with perirhinal activation [102] and thus hypothesized to potentially tap into early transentorhinal tau deposition. Decrements in this task were observed in asymptomatic presenilin-1 mutation carriers compared with non-carrier controls [103]. Borrowing from both animal and cognitive neuroscience literature is the Behavioral Pattern Separation Task (BPS-O). Older adults are more susceptible to the interference of previously learned information when differentiating similar but new information. Deficits in pattern separation have been associated with increased hippocampal CA3 and dentate gyrus fMRI activity [104], and worse BPS-O performance is associated with worse memory performance in otherwise normal older adults [105].

NOVEL PLATFORMS FOR COGNITIVE TESTING

There is currently significant interest in the use of digital technology to measure early cognitive changes in preclinical AD. While well-validated paper and pencil measures are current gold standards in clinical trials, digital technology may confer significant advantages including 1) increased ease of administration (self-administered versus rater administered platforms; remote administration; frequent serial measurements); 2) more precise and reliable scoring particularly for timed measures; and 3) potential for more extensive mineable data to examine individual variations in performance. There are numerous well-validated computerized batteries (see [106] for a review) with differing advantages (e.g., non-proprietary, web and/or tablet based application).

Many of these platforms do not simply digitize traditional tests but instead incorporate novel paradigms. For example, in the Cogstate Brief Battery, playing cards are used to measure reaction time, working memory, and incidental learning. Recently published data from 335 CN from an observational study showed longitudinal decline in Cogstate Card Identification (a measure of choice reaction time) and One Card Learning (a visual memory measure using a pattern separation paradigm) among Aβ+ compared with Aβ– [48], highlighting the ability of these computerized tests to detect early Aβ related cognitive decline among CN.

Beyond tablet and web-based interfaces for cognitive testing, commercially available digital pens can

capture extensive information about how a task is performed including information about pen strokes, pen velocity, and pen time off the page to capture "thinking" time. Digital Cognition Technologies harnesses the well-known clock drawing task, but through machine learning techniques using thousands of administrations and thousands of variables per administration, they have developed software that identifies features of performance that offer precise measurement of thinking processes, such as mental speed, time to decision-making, and organizational details. These features have the potential to move beyond simple measures of accuracy and detect subtle cognitive changes reflected in the performance of the task that may be meaningful in early AD [107]. In addition, there are smartphone based functional instruments that may detect the emergence of difficulty with everyday skills including using an ATM to perform banking tasks, managing prescription refills over the phone/computer, or navigating a telephone menu [108, 109] and thus may particularly useful for tracking clinical progression.

Finally, advances in technology have resulted in myriad sensors, trackers and monitors that collect information in real-time and offer the opportunity for passive monitoring. For example, a recent study in individuals with CN adults and those diagnosed with MCI showed that the MCI group exhibited a significant decline in their overall computer use and an increase in their day-to-day use variability in comparison with the CN adults [110]. Continuous collection of smartphone and internet use information allows for acquisition of an unprecedented amount of data. Analysis of this data using machine learning and other data driven techniques has the very exciting potential of identifying indirect and very subtle changes in behavior and provides a novel frontier for maximizing the predictive utility and precision of cognitive measurement at the level of the individual.

SUMMARY

Amyloid PET imaging has provided a unique opportunity to understand early AD changes among clinically normal individuals. Work across multiple laboratories highlights that cognitive decline is detectable among CN with abnormal levels of amyloid, and that this group is at elevated risk for clinically meaningful change at follow up. Our ability to predict which Aβ+ CN are most at risk will increase as additional biomarkers and risk factors are integrated, such as tau PET, sensitive markers of neuronal integrity, as well as genetic and lifestyle variables. Importantly, the ability to measure the pathophysiology of AD before symptoms are present, and the converging research that has shown that CN with early evidence of AD are at risk for future cognitive decline, has provided an unprecedented opportunity to explore early intervention with disease modifying strategies [111]. The results of these prevention trials will undoubtedly have a large influence on the conceptualization of AD and "normal" aging.

DISCLOSURE STATEMENT

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Underlying Biological Processes in Mild Cognitive Impairment: Amyloidosis Versus Neurodegeneration

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Abstract. The amyloid cascade hypothesis proposes amyloid-β (Aβ) as the earliest and key pathological hallmark of Alzheimer's disease (AD), but this mandatory "amyloid-first pathway" has been contested. Longitudinal studies of mild cognitive impairment (MCI) patients represent an opportunity to investigate the intensity of underlying biological processes (amyloidosis versus neurodegeneration) and their relevance for progression to AD. We re-examined our cohort of amnestic MCI, grouped according to cerebrospinal fluid (CSF) biomarkers, aiming at establishing their prognostic value for Alzheimer-type dementia and testing the hypothetical model of biomarkers sequence, based on the amyloid cascade. Our baseline population consisted of 217 MCI patients, 63% with neurodegeneration markers and 47% with amyloidosis. Within the longitudinal study-group ($n = 165$), 85 progressed to AD and 80 remained cognitively stable. Age, CSF $A\beta_{42}$, and t-Tau were identified as the best single predictors of conversion to AD. Regarding MCI classification according to the NIA-AA criteria, the high-AD-likelihood group (HL-both amyloid and neurodegeneration markers) was the most frequent (42%); followed by the Suspected Non-Alzheimer Pathophysiology group (SNAP-26%), the low-AD-likelihood group (LL-negative biomarkers-22%), and the Isolated Amyloid Pathology group (IAP-10%). Risk of progression to AD was higher in HL in relation to the LL group (HR = 6.1, 95%CI = 2.1–18.0, $p = 0.001$). SNAP and IAP groups were equivalent in terms of risk of progression to AD (IAP: HR = 2.6, 95%CI = 0.7–9.3, $p = 0.141$; SNAP: HR = 3.1, 95%CI = 1.1–9.6; $p = 0.046$), but only SNAP was significantly different from the LL group. These results support different neurobiological pathways to AD beyond the amyloid hypothesis, highlighting the alternative "neurodegeneration-first pathway" for further investigation.

Keywords: Alzheimer's disease, amyloid, cerebrospinal fluid biomarkers, mild cognitive impairment, neurodegeneration

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia worldwide and the most common neurodegenerative disease, affecting 5 to 7% of people over the age of sixty [1]. Dementia in general, and AD in particular, is considered a public global health priority considering its high prevalence, economic impact, and the associated dependency leading to social exclusion [2]. This immense burden emphasizes the urgent need for strategies that prevent or modify disease progression. As first identified by Alois Alzheimer in 1906 [3], the neuropathological hallmarks of AD are the amyloid-β (Aβ) senile plaques and tau-containing neurofibrillary tangles. The relationship between senile plaques and neurofibrillary

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tangles puzzled Alzheimer's and launched the debate about which one of these proteins represents the crucial pathogenic process in AD. The amyloid cascade hypothesis proposes that Aβ is the key pathological hallmark of AD [4]. This hypothesis succeeded and was largely supported by the discovery that the major genetic mutations in early onset familial AD are all related to an abnormal Aβ processing, further warranted by the demonstration of Aβ neuronal toxicity [5]. However, familial AD accounts for less than 1% of disease cases [6], and considering that AD is an exclusively human disease, carriers of these autosomal dominant mutations or transgenic animal models carrying the same errors have been the only available models to investigate the early pathological mechanisms or surrogates of these events (biomarkers). These studies mainly pinpoint the role of amyloid [7], though results are inevitably biased and prone to circularity, and there is no overwhelming evidence that amyloid changes represent the crucial pathogenic process in the most prevalent sporadic forms of AD. Even so, the Aβ cascade framework dominated the field for the last 25 years, and fostered conceptual biomarker models like that proposed by Jack and colleagues [8] to describe the hypothetical sequence of dynamic biomarker changes in the order of brain amyloidosis, neurodegeneration, memory deficit (mild cognitive impairment, MCI) and clinical dysfunction (dementia state). Moreover, this hypothesis promoted the development of clinical trials aiming to reduce the generation of Aβ, facilitate its clearance, or prevent the aggregation of the peptide. Most disappointingly, trials of anti-Aβ therapy in symptomatic patients did not produce clinical benefits, despite some evidence of Aβ clearance [9].

Despite the therapeutic failure, the interest in capturing the earliest stages of AD, supported by new available biomarkers of the disease like the cerebrospinal fluid (CSF) biomarkers, PET imaging and evidence of hippocampal atrophy on MRI, radically changed our diagnostic focus that has moved from the phase of dementia to prodromal or pre-symptomatic stages. The classical CSF biomarkers for AD are $A\beta_{42}$, which is found in low concentrations in AD due to brain amyloid deposition, total tau (t-Tau) at high concentrations representing cortical neuronal loss, and phosphorylated tau (p-Tau) also at high concentrations, reflecting cortical tangle formation [10]. These amyloid and neuronal injury markers have been incorporated in new diagnostic criteria, like those proposed by the National Institute of Aging-Alzheimer Association (NIA-AA) for MCI [11] or preclinical states [12] to increase the confidence that subjects with prodromal dementia have AD as the underlying cause. Longitudinal cohort studies using these criteria are becoming available and represent an opportunity to investigate the intensity of the underlying biological processes (amyloidosis versus neurodegeneration) and their relevance for the progression to dementia and AD [13–18].

With this specific purpose, we re-evaluated our cohort of amnestic MCI with available CSF biomarkers to classify subjects according to the NIA-AA MCI-sub-groups or stages, aiming to establish its prognostic value for Alzheimer-type dementia at follow-up and at the same time to test the proposed hypothetical model of biomarkers sequence conforming the amyloid cascade.

MATERIALS AND METHODS

Subjects

In 2003, we started a longitudinal assessment of patients with the diagnosis of amnestic MCI at the Dementia Clinic, Neurology Department of Coimbra University Hospital. This cohort already includes 400 MCI patients, but for this specific investigation, we only considered 217 that underwent lumbar puncture with CSF biomarkers assessment at the initial evaluation (obligatory inclusion criterion) and were enrolled until December 2016. The baseline study and follow-up protocol have been previously published [19, 20]. In brief, the patients were enrolled in a systematic way and had biannual clinical observation and annual neuropsychological and functional evaluations in order to detect progression to dementia. Cases that were followed-up with this comprehensive protocol until they developed dementia or until they had been cognitively stable for at least 2 years comprise the longitudinal study-group. This group was further dichotomized between those that were cognitively stable and those that developed dementia due to Alzheimer's disease, according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders (NINCDS-ADRDA) [21] and more recently to the 2011 NIA-AA criteria [22]. As we stated, for the biomarker-based subject classification, we used the classical CSF biomarkers for AD, operationalized according to the framework of the NIA-AA criteria for MCI and preclinical forms [11, 12, 23]. Subjects were classified in the low-AD-likelihood group if both amyloid (i.e., CSF $A\beta_{42}$) and

neuronal injury markers (i.e., CSF t-tau and/or p-tau) were normal (LL), in the high-AD-likelihood group if both amyloid and at least one neuronal injury marker were abnormal (HL), or in one of the two conflicting biomarker groups [Isolated Amyloid Pathology (IAP) group if the amyloid marker was abnormal and neuronal injury markers normal, Suspected Non-Alzheimer Pathophysiology (SNAP) group if at least one neuronal injury marker was abnormal and the amyloid marker normal].

This study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and was approved by the Ethics Board of Coimbra University Hospital. All subjects or responsible caregivers, whichever appropriate, gave their informed consent.

Clinical and neuropsychological procedures

MCI patients included in this study were of the amnestic type and the diagnosis was made in accordance with the criteria defined by Petersen [24] and more recently the framework for MCI due to AD, proposed by NIA-AA criteria [11]. Diagnostic investigation included a standard clinical evaluation, an extensive cognitive and staging assessment, standard laboratory tests, imaging studies (CT or MRI and SPECT), CSF analysis, APOE genotyping, and eventually PiB-PET (12 patients). At baseline, a neurologist completed a medical history with the patient and the caregiver and conducted a general physical, neurological, and psychiatric examination as well as a comprehensive diagnostic battery-protocol, including: 1) Cognitive instruments as the Mini-Mental State Evaluation (MMSE) [25], Portuguese version [26]; The Montreal Cognitive Assessment (MoCA) [27], Portuguese version [28]; the Alzheimer Disease Assessment Scale-Cognitive (ADAS - Cog) [29, 30], Portuguese version [31]; and a comprehensive neuropsychological battery with normative data for the Portuguese population (BLAD) [32] exploring memory (Wechsler Memory Scale sub-tests) and other cognitive domains (including language, praxis, executive functions and visuo-constructive tests); 2) Standard staging scales which provide objective information about subject performance in various domains, including the Clinical Dementia Rating (CDR) [33], Portuguese version [34] for global staging; the Disability Assessment for Dementia (DAD) [35], Portuguese version [36] for evaluation of functional status; the Neuropsychiatric Inventory (NPI) [37], Portuguese version [38] to characterize the psychopathological profile and the Geriatric Depression Scale (GDS-30) [39], Portuguese version [40] to exclude Major Depression.

All the available information (baseline cognitive test, staging scales, clinical laboratory and imaging studies) was used to reach a consensus diagnosis. A similar approach was used for follow-up annually evaluations. The baseline inclusion criteria for amnestic MCI were those proposed by Petersen [24] and were operationalized as this: 1) A subjective complaint of memory decline (reported by the subject or an informant); 2) An objective memory impairment (considered when scores on standard Wechsler memory tests were >1.5 SDs below age/education adjusted norms) with or without deficits in other cognitive domains; 3) Normal general cognition suggested by normal scores in the MMSE and MoCA using the Portuguese cut off scores [26, 41]; 4) Largely normal daily life activities, evaluated with a functional scale – DAD; 5) Absence of dementia, indicated by a CDR rating of 0.5. As exclusion criteria for enrolment we considered a significant underlying medical or neurological illness revealed by lab tests or imaging; a relevant psychiatric disease, including major depression, suggested in the medical interview and confirmed by the GDS; CT or MRI demonstration of significant vascular burden [42] (large cortico-subcortical infarct; extensive subcortical white matter lesions superior to 25%; uni- or bilateral thalamic lacunes; lacune in head of caudate nucleus; more than 2 lacunes).

The patients were observed every 6 months and clinical evaluation of progression was conducted annually, with a brief cognitive and functional status reassessment, including the MMSE, MoCA, ADAS-Cog, and the CDR. Dementia was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders – fourth edition (DSM-IV-TR) criteria [43] and AD, according to specific criteria [21, 22]. Conversion to AD required meeting clinical diagnostic criteria for probable AD and was confirmed by the coordinator of the clinical study (IS). As these criteria are not fully operational and the conversion status decision has some uncertainty and subjectivity, patients in this study were classified as having undergone conversion based on 1) Objective evidence by cognitive testing of decline to dementia using the MMSE, MoCA, and the ADAS-COG scores and qualitative evaluation (i.e., impairment of memory plus another domain); and 2) Changes in global CDR rating from 0.5 to 1 or more, confirming the cognitive profile of dementia and loss of autonomy.

Laboratory determinations

CSF samples were collected from patients as part of their routine clinical diagnosis investigation. Pre-analytical and analytical procedures were done in accordance with the Alzheimer's Association guidelines for CSF biomarker determination [44]. Briefly, CSF samples were collected in sterile polypropylene tubes, immediately centrifuged at 1800 g for 10 min at 4°C, aliquoted into polypropylene tubes and stored at –80°C until analysis. CSF $A\beta_{42}$, t-Tau, and p-Tau were measured separately by commercially available sandwich ELISA kits (Innotest, Innogenetics/Fujirebio, Ghent, Belgium), as previously described [45, 46]. External quality control of the assays was performed under the scope of the Alzheimer's Association Quality Control Program for CSF Biomarkers [44]. CSF biomarkers were classified as normal/abnormal according to previously reported laboratory reference values [47].

For APOE genotyping, peripheral blood samples were also collected into EDTA tubes and genomic DNA was isolated from leucocytes using the DNA isolation kit for mammalian blood (Roche Diagnostics, GmbH, Manheim, Germany), as described by the manufacturer. The analysis of the two polymorphisms, rs429358 and rs7412, at codons 112 and 158, respectively was performed by PCR-RFLP assay, as previously described [48].

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 20.0) (IBM SPSS, Chicago, IL). Normality of continuous variables was assessed by the Kolmogorov-Smirnov test. For normally distributed continuous variables one-way ANOVA followed either by the Bonferroni or the Games-Howell post-test was performed to assess the statistical significance of the difference between means. When continuous variables did not show normal distribution, the Kruskal-Waalis test was used, followed by the Dunn-Bonferronni post-test. Group differences between categorical variables were examined using the χ^2 test. Binary logistic regression analysis was used to identify predictive markers of conversion to AD, with conversion as dependent variable and age, gender, education, ApoE genotype, baseline MMSE, CSF $A\beta_{42}$, t-Tau, and p-Tau levels as independent variables. Survival analysis was used to assess the probability of conversion to AD in the different MCI groups. Kaplan – Meier survival curves were plotted and the survival distributions in the different subgroups were compared by the log-rank test. Survival time was calculated as the interval from the initial baseline evaluation to the diagnosis of dementia. For patients who remained non-demented, survival time was censored at the date of the last clinical assessment. A Cox proportional hazards model, corrected for age, gender, education, ApoE genotype, and baseline MMSE score was used to test the predictive ability for Alzheimer's disease-type dementia of the different MCI groups.

RESULTS

Cohort data

At baseline, 217 MCI patients were included (138 females, 79 males), with ages ranging from 40 to 85 years (mean 67.3 ± 9.4), a mean education level of 6.3 ± 4.1 years, and a mean longitudinal follow-up of 4.2 ± 3.4 (0.5–13.0) years. Demographic, clinical, genetics, and biomarker data of the baseline study population are presented in Table 1. Concerning cognitive scores, the mean MMSE score was 26.1 ± 3.3, a value above the international cut off for dementia (<24/30) [25] and the same applies to the mean values on the MoCA (17.6 ± 5.6) and the ADAS-COG (11.7 ± 6.0), both above the cut-offs for dementia, proposed for the Portuguese population, respectively <17 and >12 points [49, 50]. Regarding biological parameters, 43% were carriers of at least one ApoE ε4 allele—the typical ApoE genotyping of AD spectrum disorders [51], the mean level of CSF $A\beta_{42}$ (667 ± 310 pg/ml) was in the normal range for our center (>542 pg/ml), while t-Tau (371 ± 260) and p-Tau (50 ± 27) values were both above the respective reference values (i.e., <212 pg/ml and <32 pg/ml) [47]. Noteworthy, in this baseline study population, the percentage of patients with injury markers (63%) was higher than those with amyloidosis (47%).

Cohort classification

Using CSF biomarkers operationalized according to the framework of the NIA-AA criteria for MCI, 81 (37%) were classified in the high-AD-likelihood group (HL - subjects with both amyloid and injury markers), 22 (10%) in the IAP group, 57 (26%) in the SNAP group, and a similar number - 57 (26%) had neither amyloid nor neurodegeneration-biomarkers, being classified in the low-AD-likelihood group (LL)

(Table 2). As expected, differences were significant in terms of amyloid levels between HL and IAP versus SNAP and LL, as well as between SNAP and HL versus IAP and LL in terms of neuronal injury markers. When comparing HL with SNAP subjects, both t-tau and p-tau levels were slightly but significantly higher in the HL group and on the contrary, no difference in $A\beta_{42}$ levels was seen between HL and IAP groups.

Longitudinal study

Of the 217 MCI patients enrolled, 29 had a follow-up <2 years, 3 died, 18 were drop-outs, and 2 patients were excluded from the further analysis because, although their clinical presentation was amnestic MCI, they developed frontotemporal dementia and in fact they were carriers of C9orf72 mutation. The remaining 165 subjects with a follow-up $\geq$2 years (mean follow-up time: 5.0 ± 3.2 years) comprised the longitudinal study-group, which was further dichotomized between those that were cognitively stable in the last observation, 80 (48%), and those that progressed to dementia due to AD, 85 (52%). A logistic regression model was employed to identify the best predictors of conversion to AD. We included age, gender, education, ApoE genotype, baseline MMSE, and CSF $A\beta_{42}$, t-Tau, and p-Tau values as variables in the equation and obtained a reasonable fit (Nagelkerkes $R^2 = 0.585$), with an overall accuracy of 83%. We verified that the variables that were contributing significantly to the model classification were age ($p = 0.004$; OR = 1.099, 95% CI = 1.031 – 1.171), CSF $A\beta_{42}$ ($p = 0.001$; OR = 0.994, 95% CI = 0.991 – 0.998), and t-tau ($p = 0.003$; OR = 1.008, 95% CI = 1.003–1.013). Although ApoE ε4 was much more represented in the group of MCI patients that converted to AD (58% versus 26%; $p < 0.001$), this variable was not identified as a significant predictor of conversion to AD in our model.

The longitudinal study-group was again classified in MCI subtypes according to CSF biomarkers, with an equivalent distribution to the baseline subgrouping: HL group 69 (42%), IAP group 17 (10%), SNAP group 42 (26%), and in the LL group 37 (22%). In Table 3, we present the demographic, clinical, and genetics data of these groups. There were no significant differences regarding gender, years of education, and time of follow-up, but the HL and SNAP patients were older at baseline and at onset of the symptoms and this difference reached statistical significance in the comparison with the LL group ($p \leq 0.001$). Regarding the cognitive tests, the MMSE mean score was significantly lower in the HL group in comparison with all the other groups ($p \leq 0.001$), the MoCA mean score was also lower in this group in comparison with the LL group ($p < 0.001$) and the same applies to ADAS-Cog mean score, that was higher in HL group versus LL group ($p = 0.001$), indicating again greater cognitive impairment. Subjects in the HL group were also more often APOE ε4 carriers (63%) and more likely to progress to AD (80%), than all other biomarker groups: IAP - 47%, SNAP - 40%, LL- 14%. Conversion rates were similar in the IAP

Table 1
Demographic, clinical, genetic, and biomarker data of the study population

	MCI ($n = 217$)
Gender (M/F)	79/138
Age (y)	67.3 ± 9.4
Age onset (y)	64.6 ± 9.3
Education (y)	6.3 ± 4.1
MMSE	26.1 ± 3.3
MoCA	17.6 ± 5.6
ADAS-Cog	11.7 ± 6.0
ApoE ε4 (%)	43%
$A\beta_{42}$ (pg/mL)	667 ± 310
t-Tau (pg/mL)	371 ± 260
p-Tau (pg/mL)	50 ± 27

Data are expressed as mean $\pm$ S.D, except for ApoE that is expressed as percentage of ε4 carries. M, male; F, female; MMSE, Mini-Mental State Examination, higher scores correspond to better performance; MoCA, Montreal Cognitive Assessment, higher scores correspond to better performance; ADAS-Cog, Alzheimer Disease Assessment Scale-Cognitive, lower scores correspond to better performance.

Table 2
CSF biomarker profile of the different MCI subgroups

	low-AD likelihood	high-AD likelihood	IAP	SNAP
n (%)	57 (26%)	81 (37%)	22 (10%)	57 (26%)
$A\beta_{42}$ (pg/mL)	909 ± 269	$424 \pm 123^{***}$	$481 \pm 320^{***;\S\S\S}$	$843 \pm 220^{\gamma\gamma\gamma}$
t-Tau (pg/mL)	163 ± 44	$555 \pm 281^{***}$	$141 \pm 63^{\gamma\gamma\gamma;\S\S\S}$	$405 \pm 171^{***;\gamma\gamma}$
p-Tau (pg/mL)	30 ± 10	$67 \pm 30^{***}$	$30 \pm 9^{\gamma\gamma\gamma;\S\S\S}$	$52 \pm 21^{***;\gamma\gamma}$

Data are expressed as mean $\pm$ S.D. AD, Alzheimer's disease; IAP, Isolated Amyloid Pathology; SNAP, Suspected Non-Alzheimer Pathophysiology. $^{***}p < 0.001$ versus low-AD-likelihood. $^{\gamma\gamma}p < 0.005$ versus high-AD-likelihood. $^{\gamma\gamma\gamma}p < 0.001$ versus high-AD-likelihood. $^{\S\S\S}p < 0.001$ versus SNAP.

Table 3
Demographic, clinical, and genetic data of the MCI subgroups with clinical follow-up

	low-AD likelihood	high-AD likelihood	IAP	SNAP	*p* value
n (%)	37 (22%)	69 (42%)	17 (10%)	42 (26%)	
Gender (M/F)	13/24	32/37	6/11	12/30	0.282
Age (y)	63.5 ± 9.8	70.0 ± 7.7**	66.4 ± 8.9	70.4 ± 8.8*	0.001
Age onset (y)	60.2 ± 10.0	67.4 ± 6.9**	61.7 ± 9.3	67.8 ± 9.0*	<0.001
Education (y)	7.8 ± 4.5	6.0 ± 3.9	6.1 ± 4.0	5.4 ± 3.9	0.051
MMSE	27.3 ± 3.6	24.8 ± 3.0***	27.6 ± 2.2$^{\gamma\gamma}$	27.0 ± 3.2$^{\gamma\gamma\gamma}$	<0.001
MoCA	21.0 ± 5.0	15.8 ± 5.2***	17.9 ± 7.2	18.1 ± 5.0	<0.001
ADAS-Cog	9.0 ± 4.4	14.1 ± 4.9**	10.6 ± 5.8	10.6 ± 7.2	<0.001
ApoE ε4 (%)	24%	63%*	25%$^{\gamma}$	33%$^{\gamma}$	<0.001
Follow-up time (years)	5.0 ± 3.6	4.7 ± 3.0	5.6 ± 3.9	5.1 ± 3.1	0.584
Conversion to AD [n (%)]	5(14%)	55(80%)***	8(47%)*,γ	17(40%)*,$\gamma\gamma\gamma$	<0.001

Data are expressed as mean ± S.D, except for ApoE that is expressed as percentage of ε4 carries and conversion to AD that is expressed as percentage of patients that converted. M, male; F, female; AD, Alzheimer's disease; IAP, Isolated Amyloid Pathology; SNAP, Suspected Non-Alzheimer Pathophysiology; MMSE, Mini-Mental State Examination, higher scores correspond to better performance; MoCA, Montreal Cognitive Assessment, higher scores correspond to better performance; ADAS-Cog, Alzheimer Disease Assessment Scale-Cognitive, lower scores correspond to better performance. *$p < 0.05$ versus low-AD-likelihood. **$p < 0.005$ versus low-AD-likelihood. ***$p < 0.001$ versus low-AD-likelihood. $^{\gamma}p < 0.05$ versus high-AD-likelihood. $^{\gamma\gamma}p < 0.005$ versus high-AD-likelihood. $^{\gamma\gamma\gamma}p < 0.001$ versus high-AD-likelihood.

and SNAP groups, but significantly different from the other two groups.

Survival analysis

Since the conversion to dementia occurred at different moments of the follow-up time, a survival analysis was performed. Kaplan–Meier survival curves for the probability of conversion to AD plotted according to MCI groups are depicted in Fig. 1. The HL group was significantly associated with an estimated shorter time of conversion to AD (3.9 ± 0.4 years; 95% CI = 3.1 – 4.7) than the LL group (12.5 ± 1.4 years; 95% CI = 9.7–15.2; $p < 0.001$). Estimated time to conversion was not different between the IAP and SNAP groups (7.7 ± 1.5 years; 95% CI = 4.8–10.7 and 8.4 ± 1.0 years, 95% CI = 6.3–10.4, respectively), but was significantly different from the HL group and LL group ($p < 0.01$ and $p < 0.05$, respectively). Cox regression models, with age, gender, education, ApoE genotype, and baseline MMSE score taken into account, showed that MCI patients belonging to the HL subtype had the highest risk of progression to AD (hazard ratio 6.1, 95% CI 2.1–18.0, $p = 0.001$), compared with patients classified in the LL group (reference). MCI patients classified in the IAP and SNAP subtypes presented a very similar risk of progression to AD, that was significantly increased in comparison with the LL subtype only in the patients classified as SNAP (SNAP: hazard ratio 3.1, 95% CI 1.1–9.6; $p = 0.046$; IAP: hazard ratio 2.6, 95% CI 0.7–9.3, $p = 0.141$). Risk of progression to AD also failed to reach statistical significance difference between the HL group and the IAP ($p = 0.091$) or the SNAP group ($p = 0.062$).

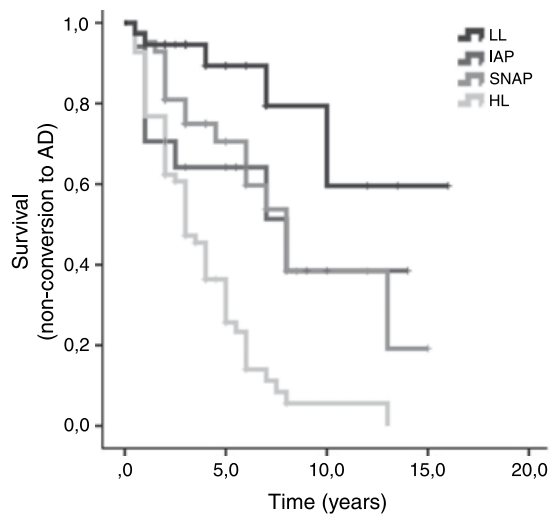

Fig. 1. Kaplan–Meier survival curves for the probability of conversion to Alzheimer's disease (AD) plotted according to the different mild cognitive impairment subgroups. LL, low-AD-likelihood; HL, high-AD-likelihood; IAP, Isolated Amyloid Pathology; SNAP, Suspected Non-Alzheimer Pathophysiology. Log-Rank (Mantel-Cox): $p < 0.001$.

DISCUSSION

In recent years, several biomarkers have been developed for AD and some of them, like the CSF biomarkers, have been incorporated in recent

diagnostic criteria defining groups or states of risk of progression to dementia. This enormous progress allowed their use by clinicians as surrogates of outcome to diagnose and potentially help to treat the disease at the mildly symptomatic stage of MCI. Our study was developed in this specific context of routine clinical practice and since we enrolled patients in a systematic way, our cohort may be considered representative of an ordinary tertiary Memory Clinic, surpassing the selection biases of investigational studies. In this context, the mean age of our cohort is lower than in community studies, although according to previously work from our group, there were no major biological distinction between younger and older MCI patients [52]. In line with the approach of a proxy routine clinical practice, we included exclusively patients with MCI (not investigational preclinical states) and because AD (and not other forms of dementia) is the main focus of discussion around the implementation of biomarkers in the referred setting, we only considered amnestic MCI subjects at baseline and those that progressed to dementia due AD at the follow-up of at least 2 years. Previous studies with cross-sectional data or longitudinal follow-up have examined the frequency of biomarker stages and its prognosis [13–18]. One uncontroversial finding of these studies, also confirmed in this work, is that subjects with both amyloid and neuronal injuries markers have the highest risk of progression, with 80% of patients in our HL group developing AD in the next 4 years. This information is highly valuable for monitoring MCI patients in clinical practice and for the selection of participants in clinical trials.

Concerning the key importance of amyloidosis in the pathogenesis of AD, namely as "an amyloid-first pathway" as suggested by the cascade hypothesis [4], the state of the art is more controversial. As we referred, this hypothesis was driven by the discovery that the major genetic mutations in familial AD are all related to an abnormal Aβ processing [6]. Likewise, a recent investigation of the dynamic of biomarkers in patients with the genetic variants of AD (autosomal mutations in *PSEN1*, *PSEN2*, and *APP* genes), highlights the deposition of amyloid as the earliest finding and the first component of a biomarker model with three sequential phases: active amyloidosis; a stable plateau of amyloid deposition; and a further stage of progressive neurodegeneration and cognitive decline [7]. This profile of "an amyloid-first pathway" in early-onset sporadic forms (<65 years) is also implicit in the longitudinal cohort of the Mayo Clinic Study of Aging [18], which assessed transition rates between biomarker states and dementia by age. This work showed that the transition rate between biomarker negative subjects to incident amyloidosis was most common in the 60–75 age-range and plateaued after the seventies. In our opinion, the strongest support for the cascade hypothesis in late-onset forms of AD, comes from studies showing that positivity of amyloid biomarkers may precede cognitive impairment by several decades [17, 53]. Returning to our results, it is remarkable that only 47% of our baseline MCI-cohort had abnormal low levels of CSF $A\beta_{42}$ and mainly in association with injury biomarkers (37%). In fact, the isolated amyloid pathology group (IAP) represented only 10% of the total cohort or the follow-up cohort and in line with other studies, these patients tended to be younger than those belonging to other pathological groups. In addition, the rate of conversion to AD of the IAP group (47%) and estimated mean time of conversion (7.7 ± 1.5) was quite similar to the "only neurodegeneration group" (SNAP), with conversion rate and estimated mean time of conversion of 40% and 8.4 ± 1.0, respectively, indicating an equivalent risk of progression to AD. However, according to the Cox Regression model, only the SNAP group had a statistically increased risk of conversion in relation of the LL group. Moreover, our logistic regression model identified both CSF-$A\beta_{42}$ and t-Tau as predictors of conversion to AD. Our interpretation of these results is that amyloidosis is intimately related to the neurodegenerative process, especially in younger patients, though the Aβ pathway is not necessary and probably is not the mostly relevant pathological event in these late-onset forms of sporadic AD.

The biomarker dynamic model of Jack and colleagues [8] and the NIA-AA criteria framework [23] propose a staging method based on the conception that biomarkers of AD follow an invariable temporal sequence in accordance with the amyloid cascade hypothesis. In line with this model, we would expect that the profile of "amyloidosis-only" or "amyloidosis plus neurodegeneration" would be dominant at the prodromal state, which was indeed corroborated in some longitudinal studies [14, 17, 18]. However, in our clinical practice we were frequently confronted with the opposite scenario: patients with typical neuropsychological and neuroimaging features of AD presenting exclusively CSF neurodegeneration biomarkers. Our results expand this empirical notion, showing that the percentage of MCI patients with injury markers within the baseline-cohort (63%) was

higher than those with amyloidosis (47%) and the same applies to the follow-up cohort—68% versus 52%, respectively. Concerning the prognosis, SNAP and IAP groups were equivalent in terms of risk of progression to AD, but only SNAP had significantly increased risk in relation to the LL group. The relevance of neurodegeneration is further confirmed by the results of the regression model, showing that t-Tau is a reliable predictor of progression to AD. This unexpected high prevalence of the SNAP group has been confirmed in studies with preclinical forms [15, 16] as well as in MCI stage [13] and remarkably, this last large MCI multicenter study, also showed that the 3-year progression rate to Alzheimer-type dementia was as high in the SNAP group (24%), as in the isolated amyloid pathology group (22%). The authors admitted several explanations for this "intriguing finding": that these subjects could have comorbidities concurring for the progression to Alzheimer-type dementia; clinical misclassification or atypical forms of AD; or that CSF $A\beta_{42}$ cut-offs may have been too conservative. None of these hypotheses fits our data: we extensively excluded other brain comorbidities (namely vascular disease); we only considered typical amnestic patients and those with a further clinical diagnosis of dementia due to AD; the mean amyloid level of our SNAP group was not transitional and in fact was quite similar to the LL group. Besides, of the 12 patients that also performed PiB-PET, we verified a total concordance between biomarkers, with 10/12 being classified as amyloid positive in both assessments and 2/10 as negative. Although we focus our discussion on recent studies [13–18], it is challenging to compare or conciliate our results with some of them and to interpret potential discrepancies. In fact, these studies are quite diverse in terms of target population (clinical versus community and/or preclinical versus MCI), the established outcomes (AD versus unspecified dementia) and they use different biomarkers that may reflect different processes or might became abnormal at different stages of the disease [54]. However, there seems to be a trend indicating that older AD patients may not exhibit a florid amyloid response. For example, in the Mayo Clinic Study of Aging [18], which is clearly aligned with the amyloid hypothesis, it is noteworthy that the sample included a large group of older individuals with "only neurodegeneration markers" (equivalent to SNAP) and that transition to dementia almost always required neurodegeneration. Thus, in our opinion, there is overwhelming information supporting different neurobiological pathways to sporadic AD beyond the amyloid hypothesis, highlighting the alternative "neurodegeneration-first pathway" for further investigation.

We believe that the added value of the present study, in addition to the strengths already emphasized along the text, is the holistic and rigorous methodology adopted to define stages and progression, obviating misclassifications; the use of neuropsychological instruments well-validated for the Portuguese population and administered by the same experienced team of neuropsychologists, which may improve reliability and diagnostic consistency; the exclusive use of CSF biomarkers, with quantitative and standardized cut-offs, which may also improve the reliability of the results. However, some limitations of the current study must be addressed. First of all, since only the amnestic subtype of MCI was considered, the generalization of the results to other forms of MCI should be cautious; similarly, results might be different when imaging biomarkers are considered; classification in biomarker sub-groups could be affected by cognitive performance and mainly by subjects-demographic characteristics, which deserves further investigation. In fact, the evidence that ApoE $\varepsilon 4$ was not identified as a significant predictor of conversion to AD in our cohort might be explained by the high prevalence of young patients, outside the age range of major ApoE influence, considering the pleiotropy effect of this polymorphism [53, 55]. Finally, to increase the strength of results we will need a longer follow-up and a more robust sample.

In conclusion, this study produced several evidences that patients with only neuronal injury markers, in our opinion, erroneously designated of Suspected Non-AD-Pathophysiology (SNAP), represent a prevalent group in the MCI stage and have a risk of progression to AD comparable to those with Isolated Amyloid Pathology (IAP). This brings together arguments for the investigation of key mechanisms of the AD pathophysiology, independently of the amyloid response. Moreover, the SNAP group seems to be the ideal target to explore new or more accurate biomarkers, including tau PET imaging, and for the development of innovative and successful therapeutic interventions.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-9908).

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Amyloid-β/Drug Interactions from Computer Simulations and Cell-Based Assays

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Abstract. Targeting the early oligomers formed by the amyloid-β (Aβ) peptide of 40 and 42 amino acids is considered one promising therapeutic approach for Alzheimer's disease (AD). *In vitro* experiments and computer simulations are often used in synergy to reveal the modes of interactions of drugs. In this account, we present our contribution to understanding how small molecules bind to $A\beta_{40}$/$A\beta_{42}$ peptides, based either on extensive coarse-grained and all-atom simulations, or a variety of experimental techniques. We conclude by offering several perspectives on the future of this field to design more efficient drugs.

Keywords: Aβ oligomers, all-atom/coarse-grained models, Alzheimer's disease, amyloid simulations, cell-based assays, drugs, *in vitro* studies

INTRODUCTION

The amyloid-β $(A\beta)_{42}$ intrinsically disordered protein, of sequence DAEFRHDSGYEVHHQKLV FFAEDVGSNKGAIIGLMVGGVVIA, produced from the amyloid-β protein precursor (AβPP) by β-secretase and γ-secretase, forms amyloid plaques by a nucleation-condensation polymerization process with nonspecific interactions. The population of the smallest pathological oligomers (dimers, trimers, hexamers, or dodecamers) is dependent on agitation, temperature, concentration, ionic strength, and sample preparation [1–6]. $A\beta_{42}$ aggregates faster and is more toxic than $A\beta_{40}$, and familial mutations make Aβ peptides either more toxic (H6R, D7N, A21G, E22G, E22Q, E22K, E22Δ, and D23N) or protective in patients (A2T and A2V in its heterozygous form) [6–20]. Designed mutations or chemical modifications at specific positions can turn on/off the aggregation and toxicity properties [21–27] by preventing amyloid formation and increasing neurotoxicity (phosphorylation of S26) [21, 22], accelerating amyloid formation (lactam bridge between D23 and K28) [6], producing less toxic fibrils (mutation L34T) [24] and oligomers (mutations L34T [25] and G33I and G33A [26]), producing

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more toxic oligomers (mutation K16N) [27], or modulating the toxicity and self-assembly (azobenzene photoswitch at G25-S26-N27 [23]).

Despite progress in the determination of Aβ fibril structures and polymorphism, characterized by 3-fold and 2-fold symmetries of $A\beta_{40}$ fibril (depending on sample origin, AD-derived brain or synthetic) and different $A\beta_{42}$ fibril structures (depending on the seeding and buffer conditions) [28–34], the structures of the monomers and small oligomers of $A\beta_{40}$/$A\beta_{42}$ alone or in the presence of drugs remain to be determined. Here, we report on our recent computational and experimental studies on this aspect. It is worth noting that at the moment, it is not possible to do accurate simulations at a molecular basis by including all complex biological entities, for instance, the neurovascular unit including astrocytes, neurons endothelial cells of blood-brain barrier, etc., as well as all known Aβ protein receptors and metabolites. The present simulations include only Aβ peptides and inhibitors in water under physiological pH, ionic strength, etc., and this is why we combined the computational studies with the studies in cell cultures.

COARSE-GRAINED AND ALL-ATOM SIMULATIONS OF DRUG/Aβ PEPTIDES

Atomistic molecular dynamics (MD) simulations in explicit solvent using the Anton computer were able to elucidate the detail of how 12 proteins of 10–80 amino acids fold into their native states within 1 ms [35] and how the cancer drug dasatinib finds its Src kinase target binding site within 15 μs [36]. This speedup in solving the equations of motion is not sufficient, however, for understanding the early-formed non-fibrillar aggregates because of the number of degrees of freedom and the timescale to be explored (days at *in vitro* conditions) [37, 38]. As a result, it is necessary to study small-size oligomers and use advanced conformational sampling methods, such as replica exchange molecular dynamics (REMD) [39] or simulated tempering [40] coupled either to atomistic protein force fields (e.g., CHARMM22*, AMBER99sb-ildn, and OPLS-AA) [35, 42, 43] with water models (e.g., TIP3P) or coarse-grained (CG) models [38, 44]. For CG models that eliminate many unimportant degrees of freedom and replace groups of atoms by a single bead, there are, however, two main issues: 1) how to derive effective potentials that maintain the all-atom physical behavior in a water environment [44]; and 2) how to account for the hydrodynamics effects if we use an implicit solvent [45, 46].

We recall that the six-bead CG OPEP (Optimized Potential for Efficient peptide structure Prediction) model (an all-atom backbone with CG side-chains) and force field we developed have been extensively used with success on many proteins [47–53] and protein complexes [54]. Also, we were the first to observe β-barrels [55] during self-assembly of amyloid peptides that were validated by X-ray micro crystallography and all-atom simulations [44, 56]. It is worth noting that atomistic force fields perform well on proteins with well-defined and stable 3D structures, but provide different equilibrium ensembles on intrinsically disordered proteins, so the best force field remains to be determined [57–61].

Many drug molecules have been screened against Aβ aggregation using computer simulations [62–67]. In this account, we focus on four systems, and in what follows, the N-terminal spans residues 1–16, the central hydrophobic core (CHC) spans residues 17–21, the loop region covers residues 22–29, and the C-terminal region covers residues 30–42.

1,4-naphthoquinon-2-yl-L-tryptophan (NQTrp)

NQTrp was found to reduce the toxicity of wild-type (WT) $A\beta_{42}$ oligomers toward a cultured neuronal cell line and transgenic AD Drosophila model. The nuclear magnetic resonance (NMR) structure of NQTrp bound to $A\beta_{12\text{-}28}$ monomer at a molar ratio 0.5:1 showed three dominant binding sites between NQTrp and the $A\beta_{18\text{-}21}$ region, but no nuclear Overhauser effects were observed [68]. CG OPEP simulations of $A\beta_{17\text{-}42}$ trimer in solution, followed by all-atom docking and MD simulations, showed that the curcumin, EGCG, 2002-H20, resveratrol, and NQTrp drugs have more favorable binding energies for the most populated predicted Aβ structures than for the fibril state, and NQTrp can have multiple binding modes even within a given pocket [69]. Moving to CHARMM22* REMD simulations of $A\beta_{1\text{-}28}$ dimer with two NQTrp, and NMR experiments with different $A\beta_{1\text{-}28}$ to NQTrp ratios, our results showed that NQTrp has no "binding-site" type interaction [70].

Epigallocatchine gallate (EGCG)

EGCG was reported to redirect the aggregation of the $A\beta_{42}$ peptide into off-pathway oligomers [71]. We performed an all-atom REMD study on $A\beta_{42}$

dimer with the OPLS-AA force field and TIP3P solvent in the presence and absence of EGCG molecules with a molar ratio 2:10 (Aβ: EGCG) as used experimentally [72]. Upon EGCG binding, the bend, turn, coil, and helix contents remain constant and only the β content is reduced from 8% to 4%, but the β-strand reduction is significant within residues 1–16 (varying from 10% to 1%), the CHC and residues 39–42 (varying from 20% to 5%). Interestingly with EGCG, the CHC/CHC, and C-terminal/C-terminal interactions observed in pure $A\beta_{42}$ dimer are greatly reduced, leading to a significant increase of 8% of the cross-collision sections of $A\beta_{42}$ dimer. The EGCG molecules are buried in the interface between the peptides and bind essentially to the hydrophobic residues of the CHC and C-terminal region by van der Waals interactions, and to the N-terminal D1, E3, R5, D7, and E11 residues by hydrogen-bonds, consistent with the picture derived from isothermal titration calorimetry [73]. Overall, this simulation on $A\beta_{42}$ dimer with 10 EGCG predicts that 5% of free $A\beta_{42}$ monomers can associate to larger toxic and non-toxic aggregates. Whether the association of two possible drug candidates or the use of larger drug molecules can prevent the formation of larger $A\beta_{42}$ aggregates was explored in the next two cases.

SEN304-INH3

The SEN304 (d-[(chGly)-(Tyr)-(chGly)-(chGly)(mLeu)]-NH(2), with D chirality, ch for cyclo-hexyl and m for a N-methyl group) inhibitor [74] and the penta N-methylated peptide 3 (INH3) [75] inhibitor were tested separately on amyloid aggregation and toxicity using multiple experimental techniques. Our computational goal was to determine whether these designed molecules aimed at targeting the $A\beta_{16\text{-}22}$ and $A\beta_{32\text{-}37}$ regions, respectively, could act in synergy, stabilize the monomer of $A\beta_{40}$, and prevent its aggregation. To this end, we present unpublished results of the REMD simulation of $A\beta_{40}$ monomer with two SEN304 and two INH3 molecules. A total of 64 replicas ranging from 300 to 400 K was used, each replica for 400 ns, using the CHARMM22* and TIP3P water force fields, starting from a randomly chosen configuration and orientation of the five molecules. The procedure described in [70] was used to obtain all necessary force field parameters for SEN304 and INH3. The first 50 ns of each replica were excluded from analysis.

The convergence of the simulations was assessed by several metrics (data not shown). Fig. 1A reports the secondary structure of the $A\beta_{40}$ peptide along the sequence at 315 K. This temperature was selected because it is near physiological temperature. Our results show that the presence of the four drug molecules lead 25% of β-strand at residues E11 and V12, and a high α-helix probability spanning the CHC (with a maximum of 25%) and the residues 30–36 (with a maximum of 67% for residue I32). Figure 1B shows the distribution of free Aβ monomer as a function of the minimal distance between any heavy atoms of Aβ and any heavy atoms of the four drugs. Using a standard cut-off distance of 0.35 nm, we see, in contrast to the simulations of $A\beta_{1\text{-}28}$ dimer with two NQTrp, that the population of free $A\beta_{40}$ monomer is close to zero, indicating a tight binding between the receptor and the drugs. Figure 2 shows 20 clusters obtained from the backbone dihedral angle principal component analysis (dPCA) analysis [72] of the Aβ peptide. First, all 20 clusters differ in the conformations of the Aβ monomer. The states S1, S3, S12, and S20 are essentially random coil, states S2, S5, S7, S8, S9, and S10 display some α-helices at different positions, while states S4, S11, S14, S15, and S16 display mixed α-β configurations. Also, all clusters differ in the positions and orientations of the four drugs. To get a better understanding of the binding, Fig. 3 shows the contact probability map between the $A\beta_{40}$ monomer and the drugs. Both drugs are very mobile and bind preferentially to the CHC and residues 30–36, but transient interactions are also observed with the residues V24 and N27 of the loop and C-terminal residues (F4, Y10, and V12). SEN304 was designed based on modifying the self-recognition element $A\beta_{16\text{-}20}$ sequence [74]. While it does indeed bind to this region, it also binds to residues 30–36.

Chignolin

The last system, for which we present unpublished atomistic REMD results, is $A\beta_{40}$ with four chignolin peptides. Our selection of chignolin (GYDPETGTWG) was motivated by two experimental results. First, the phage-display selected protein ZAβ3 of 58 amino acids inhibits $A\beta_{40}$ fibrillation at stoichiometric concentrations, with the bound $A\beta_{40}$ conformation featuring a β-hairpin comprising residues 17–36 [76]. Also, the complex interface displays a four-stranded β-sheet consisting of the $A\beta_{17\text{-}36}$ region and the residues 15–19 of the two ZAβ3 proteins. This β-hairpin covering 17–36 has been proposed as an intermediate conformation on

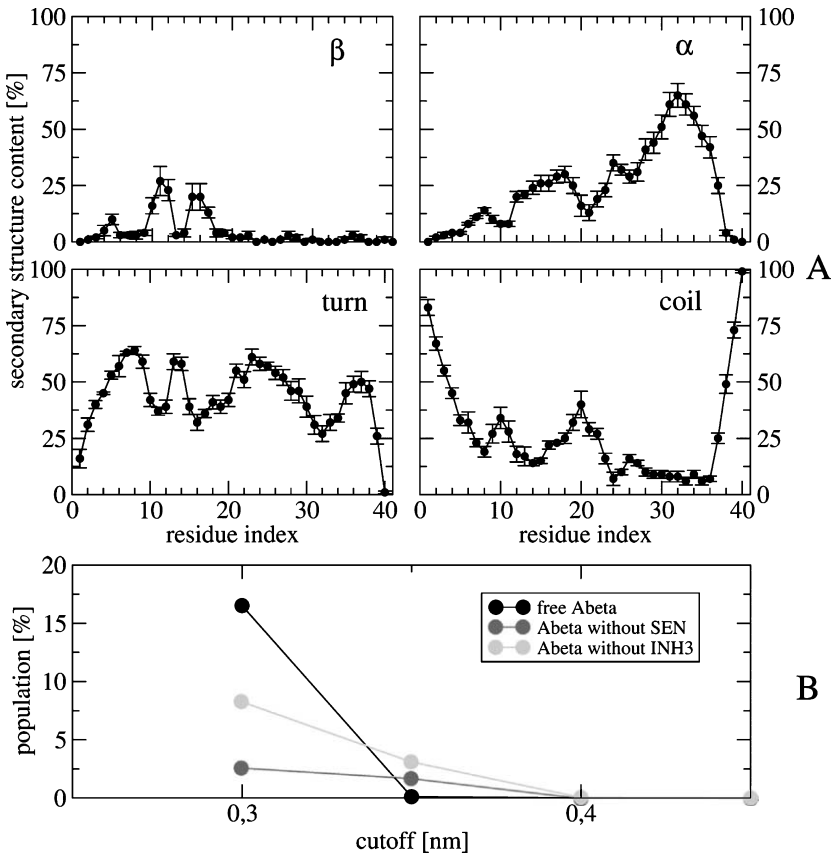

Fig. 1. A) Secondary structure contents (in %) along residues of the $A\beta_{40}$ obtained from 50–400 ns of the REMD trajectory at 315 K. The average values are: 4.6% for β-strand, 24.2% for helix, 43.8% for turn and 27.2% for coil. Error bars are also shown. B) Populations of the free monomer $A\beta_{40}$ (black), $A\beta_{40}$ in contact with two INH3 molecules but free from two SEN (red), $A\beta_{40}$ in contact with two SEN drugs but free from two INH3 (green).

the pathways to amyloid fibrils [6, 77–79]. Second, our idea was to find a peptide inhibitor that would stabilize the β-hairpin in Aβ by favoring a four-stranded β-sheet in the complex, form a β-hairpin like structure alone in aqueous solution, and be not cytotoxic. Looking at the literature, we find that the 10-residue chignolin is monomeric in aqueous solution, forms a β-hairpin like conformation by NMR, and is one of the most stable peptides [80]. Atomistic simulations have shown that many force fields can predict the correct fold and thermal stability of chignolin in explicit solvent [81–83].

REMD of the complex was performed with 64 replicas spanning 300–480 K, each replica for 400 ns, using CHARMM22* and TIP3P model. All replicas start from a designed structure shown in Fig. 4, where the initial conformation of $A\beta_{40}$ is extracted from a predicted β-hairpin spanning residues 17–36 obtained from our previous $A\beta_{40}$ dimer simulations [79], the initial configuration of chignolin is the NMR structure [80] and the four chignolin molecules are randomly orientated in a water box of approximately 200 nm^3, resulting in a Aβ concentration of 8.3 mM. Figure 4 shows the free energy landscape (FEL) of the Aβ peptide in the complex at 315 K, using the trajectories 50–400 ns and dPCA analysis (with the first two V1 and V2 components). We have checked that the results are converged and are independent of the time windows using for analysis 50–225, 225–400, or 50–400 ns. The FEL displays eight minima (S1–S8) with populations varying between 20.4% (S1) and 4.5% (S8). In all states, the β-hairpin architecture spanning residues 17–36 is formed, which is otherwise lost in the absence of chignolin (data not shown). Additionally, in states S1, S3, S4, S6, and S7 the C-terminal region makes contacts with the N-terminal region, resulting in 3-stranded β-sheets. All chignolin peptides retain the β-hairpin conformation most of the time, as shown in the secondary structure composition at 315 K in Fig. 5.

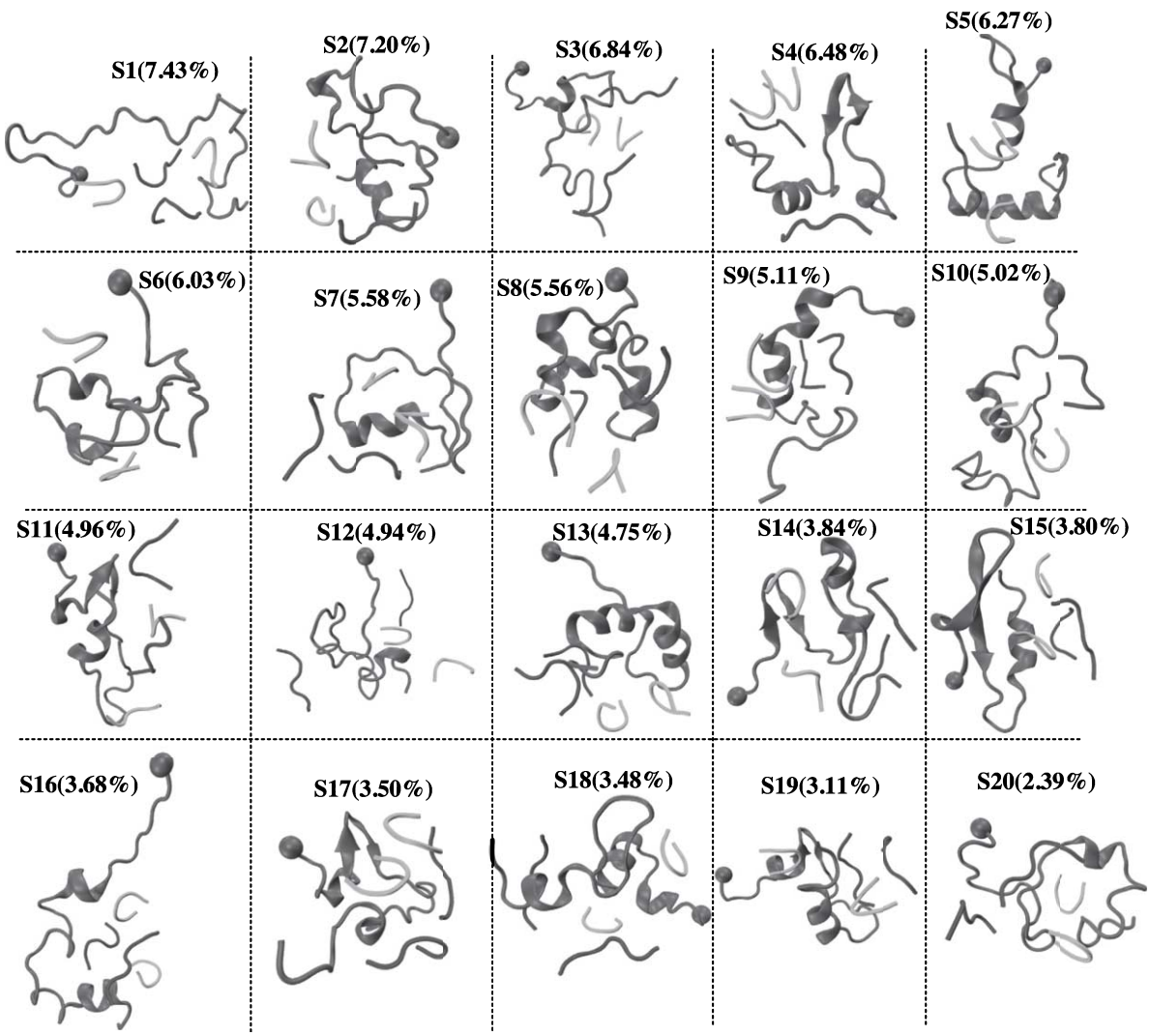

Fig. 2. Representative structures and populations of 20 conformational states of the $A\beta_{40}$ monomer (red) in the presence of two SEN (green) and two INH3 molecules (blue). The ball indicates the first residue of $A\beta_{40}$.

Consequently, the complex is stabilized by interactions between the β-strands of chignolin with those of the N-terminal region (residues 4–6), CHC region, and C-terminal region (residues 32–40) of Aβ peptide, as shown by the intermolecular contact map (Fig. 6).

DRUG TESTING USING CELL-BASED ASSAYS

To complement the possible therapeutic effects of the drugs studied by computational methods, we used SH-SY5Y cells acutely treated with $A\beta_{42}$, and SH-SY5Y_{695} cells overexpressing Aβ [84], as *in vitro* models to mimic the neurotoxic effects of Aβ in AD. The MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) cell viability assay is extensively used in studies measuring Aβ toxicity. Healthy cells reduce MTT, but this metabolic process is decreased when SH-SY5Y cells are treated with $A\beta_{42}$. We used this assay to evaluate whether cells treated with 1 μM EGCG, SEN304, INH3, chignolin, and NQTrp alleviated $A\beta_{42}$ toxicity. EGCG and SEN304 significantly reduced $A\beta_{42}$ toxicity, ($p < 0.0001$) compared to cells treated with 1 μM $A\beta_{42}$, while INH3, chignolin and NQTrp had no significant effect (Fig. 7). These results correlate very well with the capacity of these compounds to inhibit fibril formation of $A\beta_{40}$ as assessed by

Thioflavin T (ThT) fluorescence and atomic force microscopy (AFM), SEN304 and EGCG inhibit fibril formation in a dose dependent manner, whereas chignolin, INH3 and NQTrp show no significant inhibition (see Supplementary Figures 1–3). Note we previously reported that only very high concentration of NQTrp might partially rescue cells from Aβ, indicating that the reported anti-AD activity of NQTrp in *in vivo* models has to involve another mechanisms [85].

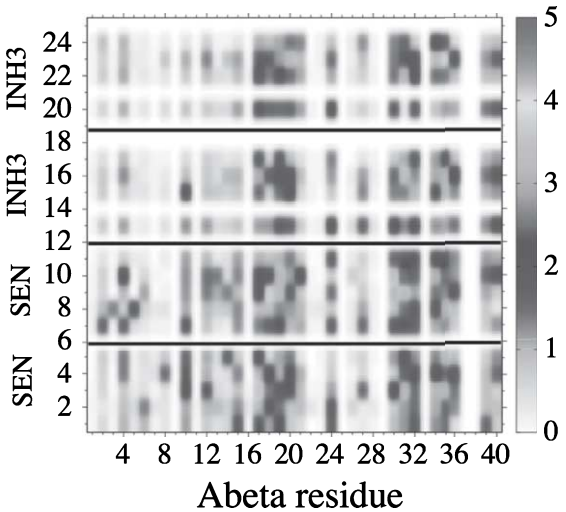

Fig. 3. Probability (in %) of forming contacts between residues of the monomer $A\beta_{40}$ (horizontal axis) and those of four drugs (vertical axis). Residue indices of drugs are (1–5: first SEN), (7–11: second SEN), (13–18: first INH3), and (20–25: second INH3). A contact is considered if the shortest distance between heavy side-chain atoms of the $A\beta_{40}$ and those of drugs is smaller than 0.45 nm.

SEN304 and EGGC were further investigated by the cell viability assay at a range of concentrations (Fig. 7). Both EGCG and SEN304 reduced $A\beta_{42}$ toxicity in a dose-dependent manner. EGCG seems to be the most potent ($EC_{50} = 0.8\ \mu M$) as it restores MTT reduction to 100% in cells treated with only 2.5 μM drug; SEN304 ($EC_{50} = 2.5\mu M$) also restored MTT reduction to 100% when cells were treated with 10 μM of this drug, in agreement with previous work [74]. Both drugs were tested without $A\beta_{42}$ (dotted lines Fig. 7B) to ensure that the compounds are not toxic.

Next, we used the Meso Scale Discovery system (MSD) to assess whether 5 μM SEN304 or EGCG affected AβPP processing and secretion in SH-SY5Y_{695} cells. SEN304 decreased the amount of $A\beta_{40}$, $A\beta_{42}$, and sAβPPβ below 80% compared to the non-treated cells. It also increased the signal of sAβPPα to ∼115%. EGCG had no effect on $A\beta_{40}$ and

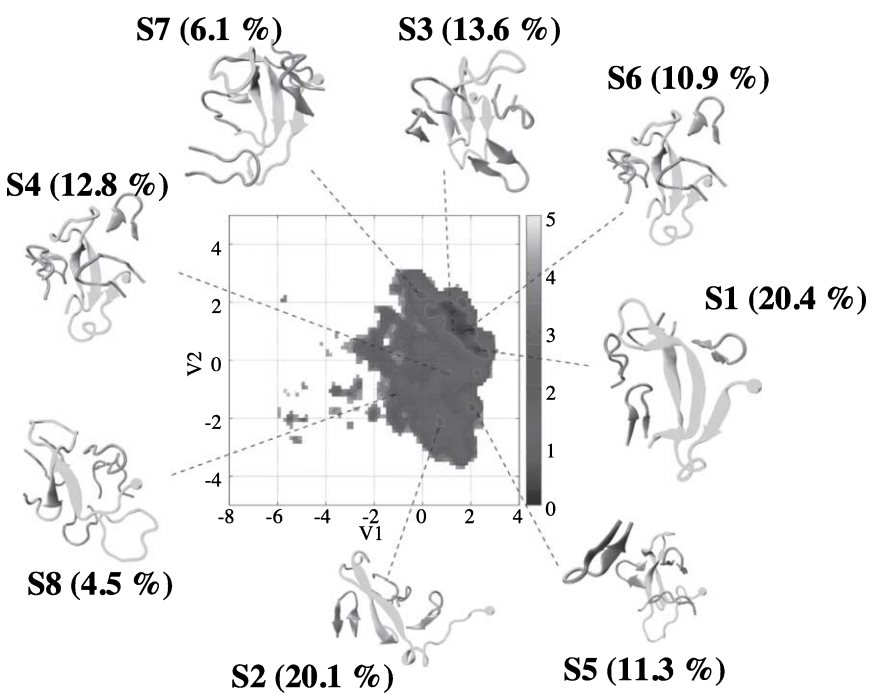

Fig. 4. Free energy landscape (in kcal/mol) of $A\beta_{40}$ (green) in the presence of four chignolin peptides (orange) as a function of the first two principal components (V1 and V2) obtained from PCA on the backbone dihedral angles of $A\beta_{40}$. Shown are the centers of the free energy minima.

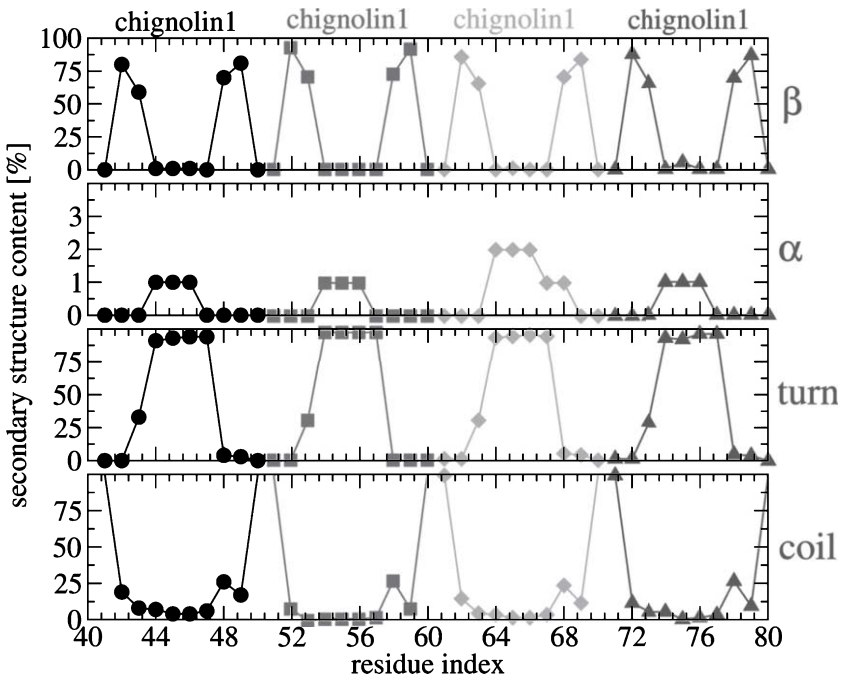

Fig. 5. Secondary structure contents (in %) along residues of the four chignolins obtained from 50–400 ns of the REMD trajectory at 315 K.

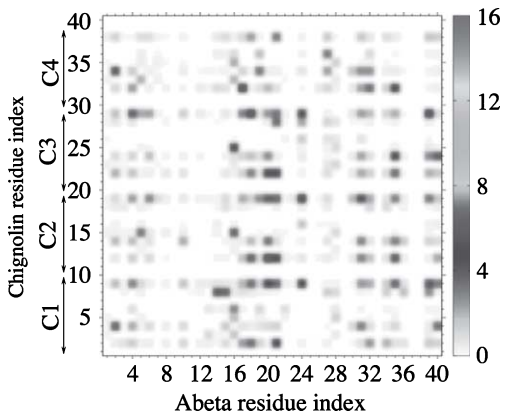

Fig. 6. Intermolecular side-chain–side-chain contact probabilities (in %) of $A\beta_{40}$ with four chignolins (denoted as to C1-C4) at 315 K. A contact is considered if the shortest distance between two heavy side-chain atoms is smaller than 0.45 nm.

$A\beta_{42}$, slightly increased sAβPPα and had no effect on sAβPPβ (Fig. 8).

We studied whether the drugs could also decrease oxidative stress caused by $A\beta_{42}$, as some studies suggest that an increase in oxidative stress could be one of the factors preceding AD and that it could also promote Aβ production [86, 87]. First, we performed a DCFH assay which allowed us to assess whether SEN304 or EGCG decreased the increase of reactive oxidative species (ROS) in cells treated with 5 μM Aβ (Fig. 9A). DCFH measures different types of ROS species, including H_2O_2, hydroxyl radicals (•OH), and nitrile radicals ($\bullet NO_2$) [88]. 5 μM EGCG completely abolished oxidative stress caused by 5 μM $A\beta_{42}$, whereas SEN304 had no effect. To complement the measurements of oxidative stress we measured the ratio of glutathione (GSH)/ glutathione disulphide (GSSG). Glutathione is mostly found in its reduced form GSH, but when cells are exposed to oxidative stress, GSH is oxidized to GSSG. Thus, the ratio GSH/GSSG is a good measure of oxidative stress. EGCG partially restored GSH/GSSG ratio compared to cells treated with 5 μM $A\beta_{42}$ (Fig. 9B).

CONCLUSIONS AND PERSPECTIVES

We have reported *in silico*, biophysical, and cell assays for four drug molecules: NQTrp, SEN304, EGCG, and INH3, and one plausible inhibitor, the chignolin peptide. Our simulations and experimental results on NQTrp indicate that this compound is not appropriate for blocking Aβ aggregation and toxicity [70].

EGCG is the main catechin (antioxidant flavonoid) found in green tea. Several *in vitro* and *in vivo* studies have pointed to EGCG as a potential treatment for AD. For instance, EGCG inhibits Aβ toxicity in PC12 and neuroblastoma mice cells when measured with

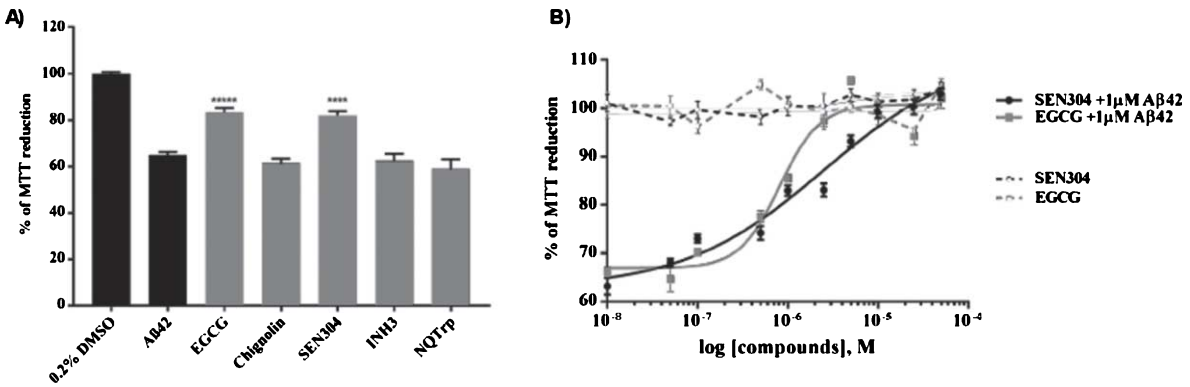

Fig. 7. A) MTT screening of drugs. SH-SY5Y cells were treated with a mixture of the indicated drug and $A\beta_{42}$ with both at 1 μM in triplicate. After 24 h incubation at 37°C, an MTT assay was performed to measure the cell metabolism (% of MTT reduction) and each compound's ability to attenuate toxicity caused by $A\beta_{42}$. Error bars represent standard error of mean (SEM). $^{****}p < 0.0001$. B) MTT dose-response curves of SEN304 and EGCG. SEN304 and EGCG were added to SH-SY5Y cells at different concentrations ranging from 50 μM to 10 nM along with 1 μM $A\beta_{42}$ (solid lines). Compounds were also incubated at the same concentrations without $A\beta_{42}$ (dotted lines). Data from the drug response curves of all the compounds were fitted using a 4PL dose response model to give their EC50 values. Error bars represent SEM, $n = 3$.

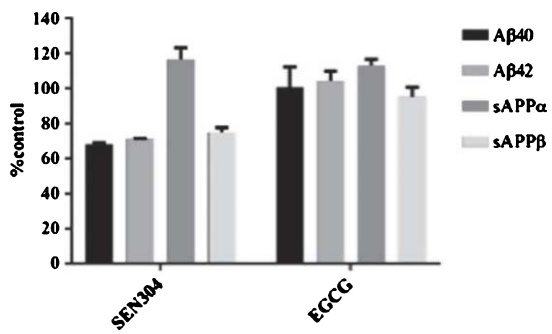

Fig. 8. SH-SY5Y APP_{695} cells were incubated with 5 μM of EGCG or SEN304 for 24 h. After incubation, 25 μl of media from the cells was placed in the MSD immunoassay V-Plex Aβ peptide panel for measuring secreted $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ concentrations. Another 25 μl was added to the sAβPPα/sAβPPβ kit to measure secreted sAβPPα and sAβPPβ concentrations. Data are represented as the mean of each parameter evaluated, $n = 2$ and the error bars represent standard deviations.

MTT [89, 90]. One possible mechanism of action for EGCG is as an aggregation inhibitor, by redirecting Aβ aggregation to off-pathway oligomers that are not as toxic as on-pathway oligomers [71]. In primary neurons from Swedish mutant APP mice, it was found that EGCG activates AβPPα processing [91]. Our data shows that EGCG increased MTT reduction in cells treated with $A\beta_{42}$, but did not activate sAβPPα, or decrease $A\beta_{40}$ or $A\beta_{42}$ in the MSD immunoassay.

EGCG has been previously studied as a possible treatment for AD. Our data agrees with previous studies that suggest that EGCG inhibits Aβ fibril formation and Aβ toxicity when measured with MTT in a concentration dependent-manner [92]. Moreover, other groups have studied the increase of ROS in cells treated with Aβ fragments and have also found that EGCG significantly decreased the ROS signal [93]. Our data also shows that EGCG increased the ratio of GSH/GSSG in cells treated with Aβ. The effect of EGCG decreasing oxidative stress could be due to its action as ROS scavenger, but studies also suggest that it could be because it promotes the production of glutathione [94].

SEN304 is an optimized peptide based on the site recognition sequence (SRS) KLVFF corresponding to residues 16–20 of Aβ [95]. This sequence was identified as key for Aβ-Aβ interactions [96]. For this reason, this SRS was used as a template to design Aβ aggregation inhibitors, including SEN304. The peptide works by promoting a rapid aggregation of monomers in an alternative aggregation mode that produces larger, but less toxic aggregates [74, 97]. Our results agree with previous observation that SEN304 attenuates $A\beta_{42}$ toxicity in SH-SY5Y cells assessed by MTT [74]. Surprisingly for an aggregation inhibitor, SEN304 also decreases production and secretion of $A\beta_{40}$, $A\beta_{42}$, and sAβPPβ and increases sAβPPα. There are no previous studies, to our knowledge, that investigated alternative mechanisms of action for SEN304, such as affecting AβPP processing. However, the 6E10 antibody, the captured antibody for the MSD $A\beta_{42}$ panel, was previously used by Amijee et al. as the monoclonal antibody for a single-site ELISA to assess the effect of SEN304 on Aβ oligomer formation [74]. They found

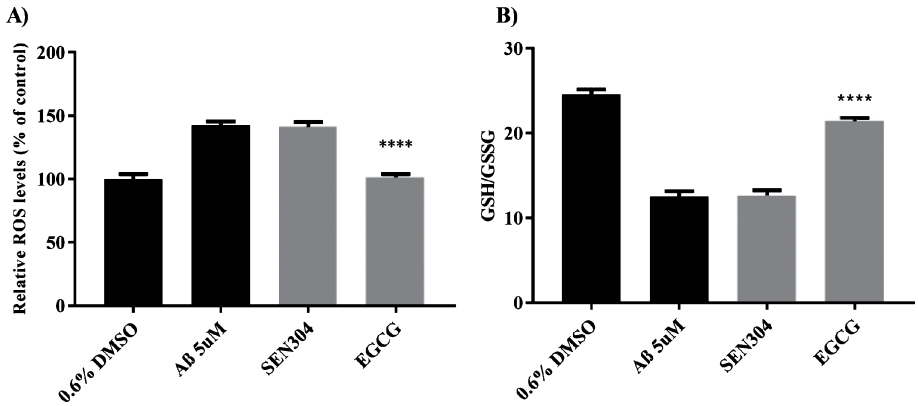

Fig. 9. A) ROS levels measured using DCFH-DA. A) SH-SH5Y cells were incubated with 5 μM of each of the primary screening hits and 5 μM of $A\beta_{42}$ 5 μM. Controls were treated with 0.6% DMSO or 5 μM $A\beta_{42}$. After 24 h incubation at 37°C, DCFH-DA assay was performed to measure ROS levels. B) GSH/GSSG ratio was measured using GSH/GSSG- Glo™. After 24 h incubation at 37°C GSH/GSSG- GloTM assay was performed to measure GSH/GSSG ratio. $n = 3$ and the error bas represent (SEM). Data was analyzed using ANOVA (one-sided) Dunnett's *post hoc* test. $^{****}p < 0.0001$.

that SEN304 reduced the Aβ signal in the ELISA assay because SEN304 binds to Aβ monomers and oligomers. Considering this, and the fact that both SEN304 and 6E10 bind to the same Aβ region, it is possible that the decrease in $A\beta_{40}$ and $A\beta_{42}$ in the MSD assay is because SEN304 is already bound to the SRS KLVFF blocking the binding of 6E10. However, this does not explain why SEN304 decreases sAβPPβ and increases sAβPPα, as this would require affecting secretase activity. SEN304 may therefore have an additional mechanism of action besides the one it was initially designed for.

We have combined INH3 and SEN304 inhibitors in simulations. The absence of a clear pattern between Aβ/drug interactions indicates that the two molecules compete with each other, despite being designed to bind to different regions of Aβ. Hence, we would not expect any favorable synergy in retarding $A\beta_{40}$ oligomerization compared to the effect of each drug taken separately. We also tested for the first time whether the chignolin peptide could be suitable as an inhibitor. Simulations report that Aβ retains the β-hairpin in the presence of chignolin, while experimental studies indicate that this molecule is not able to stabilize the β-hairpin in the monomer, and prevent Aβ aggregation and toxicity. This highlights the difficulty in designing new drugs in a milieu that simplifies cells. In a recent viewpoint, we have provided some reasons explaining why research on Aβ fails to give new drugs for Alzheimer's disease [98]. These include, but are not limited to, differences between *in silico*, *in vitro*, and *in vivo* concentrations, the use of $A\beta_{40}$ or $A\beta_{42}$ peptides while AD brain plaques consist of many Aβ peptide sequences [99] and a stoichiometry that varies with the severity of the disease [100], and the neglect of the pas de deux between Aβ and the tau protein disease [101]. As recently stated by two recent articles [98, 102], it is also time to stop AD before it starts by primary prevention human trials aimed at investigating drugs designed to treat AD before brain pathology.

METHODS

Materials

The inhibitors were obtained as follows: EGCG (Sigma), SEN304 (purchased from Peptide Protein Research Ltd), NQTrp (synthesized as described in [85]), INH3 (from rPeptide) and chignolin (purchased from Genecust). $A\beta_{42}$ was purchased from rPeptide. The GSH/GSSG-Glo™ kit was purchased from Promega. V-Plex plus Aβ peptide Panel 1 (6E10) kit and sAβPPα/sAβPPβ kit were purchased from MesoScale Discovery. SHSY5Y cells were acquired from the European Collection of Authenticated cell cultures (ECACC). SH-$SY5Y_{695}$ cells were kindly donated by Prof. Nigel Hooper.

$A\beta_{42}$ preparation

$A\beta_{42}$ lyophilized powder was dissolved in hexafluroisopropanol (HFIP) at a concentration of ∼1 mg/ml and vortexed in three cycles of 30 s to mix. After adding HFIP, the peptide was incubated at room temperature for 1 h to dissolve it completely

and then aliquoted into 20 Eppendorf tubes of 50 μl (50 μg) each. Aliquots were lyophilized by streaming gaseous N_2 to evaporate HFI, leaving the peptide coated onto the wall of the tube. The resulting lyophilized peptide aliquots were stored at –20°C until required. Anhydrous DMSO was added to the lyophilized aliquots of $A\beta_{42}$ to obtain a concentration of 1 M. As DMSO is toxic for SH-SY5Y cells when it is present in concentrations above 1%, this stock was diluted in non-supplemented Opti-MEM without phenol medium to obtain a final concentration of 1 μM $A\beta_{42}$, 0.1% DMSO or 5 μM $A\beta_{42}$ 0.5% DMSO, when added to the cells.

Cell culture

SH-SY5Y and SH-SY5Y_{695} human neuroblastoma cells were maintained in MEM Earle's medium/ Ham's F12 (1:1) supplemented with 10% fetal bovine serum (FBS), L-Glutamine (L-Q), 1% penicillin-streptomycin and 1% non-essential amino acids (n-aa). The cells were cultured in tissue flasks and incubated at 37°C, 5% CO_2 atmosphere. When cells reached ~80% confluency, they were either harvested for cell viability assays or passaged into new flasks.

Drug preparations

For MTT, an $A\beta_{42}$-drug mixture was prepared by adding $A\beta_{42}$ in non-supplemented Opti-MEM to achieve a concentration of 1 μM $A\beta_{42}$, 1 μM drug, and 0.2% DMSO. For both DCFH and GSH/GSSG assays, drugs were tested at a concentration of 5 μM with 5 μM $A\beta_{42}$ and 0.6% DMSO as controls in triplicates. For MSD assays, 1 mM stocks of drugs were diluted in Opti-MEM to achieve a final concentration of 5 μM drug and 0.5% DMSO. For dose response curves, 10 concentrations of drugs, ranging from 50 to 0.01 μM, were tested by incubating the cells with 1 μM $A\beta_{42}$. The drugs in different concentrations without $A\beta_{42}$ were used as controls. All tested groups were incubated in triplicate.

MTT assay

100 μl of SH-SY5Y cells was seeded in 96-well plates at 3×10^4 cells/well in MEM Earle's medium/ Ham's F12 (1:1) supplemented with 10% FBS, 1% L-Q, and 1% n-aa penicillin-streptomycin. The plates were incubated overnight at 37°C with 5% CO_2 to allow cell adherence. After the incubation time, media was carefully removed from each well and 100 μl of the $A\beta_{42}$-drug mixture was added to wells in triplicate using reverse pipetting. The plate was returned for incubation for 24 h at 37°C with 5% CO_2. After incubation, the MTT assay was performed. Firstly, 50 μl of media was removed and 10 μl of sterile MTT (2.5 mg/ml) was added to each well. The cells were incubated for 3 h at 37°C with 5% CO_2. Then 100 μl of acid-isopropanol (stock solution 100 ml of isopropanol and 398 μl of HCl 37%) was added. To allow solubilization of the formazan crystals, the bottom of the wells was scraped with the micropipette tip and mixed thoroughly. The plates were covered with foil and placed in a plate-shaker for 15 min. The absorbance of the plates was measured using a Tecan Infinite M200 Pro microplate reader at 570 nm.

Percentage of MTT reduction (cell viability) was calculated as:

$$\%\ \text{MTT reduction} = \frac{\text{X-A}}{\text{B-A}} \times 100\%$$

where X is the absorbance value of each well, A is the mean absorbance of the blank (buffer only), and B is the mean absorbance of the non-treated cells.

MSD assay

SH-SY5Y APP_{695} cells were seeded at a 5×10^4 cells/well in a 96-well plate. Cells were incubated overnight at 37°C, 5% CO_2, to allow cell adherence. Media was replaced with Opti-MEM non-supplemented media containing drugs at 5 μM with 0.5% DMSO. Treated cells were returned to the incubator for another 24 h. Aβ peptides ($A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$) and the soluble AβPP fragments (sAβPPα/β) were measured from the cell-media using the V-Plex Aβ panel (6E10) kit and the AβPPα/sAβPPβ multiplex kit, from Meso Scale discovery (MSD), respectively.

DCFH assay

100 μl of SH-SY5Y cells at 3×10^5 cells/ml per well were seeded in MEM Earle's medium/ Ham's F12 (1:1) supplemented with 10% FBS, 1%L-Q, and 1% n-aa penicillin-streptomycin in a 96-black plate. The cells were incubated overnight at 37°C with 5% CO_2 to let the cells attach to the bottom of the black 96-well plate. The cells were incubated for another 24 h with $A\beta_{42}$-drug mixtures. A mother stock of DCFH at 100 M in DMSO was dissolved in PBS to achieve a concentration of 100 μM DCFH and 0.1%

DMSO. The media was replaced from all wells with the diluted DCFH solution and the plate was returned to the incubator for 30 min. Afterwards, each well was washed with 200 µl of PBS to eliminate fluorescence coming from the media and ensure the measured fluorescence was coming from the cells only. The fluorescence was read using a Tecan Infinite M200 at an excitation of 480 nm and 530 nm emission. Data was normalized using the following formula:

$$\%\ \text{DCF fluorescence} = \frac{\text{X-A}}{\text{B-A}} \times 100\%$$

where X is the fluorescence value of each well, A is the mean fluorescence of the blank (buffer only), and B is the mean fluorescence of the non-treated cells.

GSH/GSSG

100 µl of SH-SY5Y cells at 1×10^5 cells/ml were seeded in a 96-white well plate using MEM Earle's medium/Ham's F12 (1:1) supplemented with 10% FBS, 1%L-Q, and 1% n-aa penicillin-streptomycin and 1% L-Glutamine, and incubated overnight at 37°C with 5% CO_2. The media was the replaced with 100 µl of non-supplemented Opti-MEM containing its respective concentration of drug or control and incubated for another 24 h. For this assay, there were two sets in triplicate for each of the treatments: one set was used to measure total glutathione and one set to measure oxidized glutathione. Glutathione was measured using a GSH/GSSG-Glo™ assay from Promega. The assay was performed as per manufacturer's instructions. The plate was read in a Promega Glo-Max-Multi Detection system. The data was normalized to GSH/GSSG ratio using the following formula

$$\text{GSH/GSSG} = \frac{\text{T-O}}{\text{O/2}}$$

where T is total glutathione relative units and O is oxidized glutathione. The oxidized concentration of glutathione was divided by two, because, when a mole of GSSG is reduced, it produces two moles of GSH.

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SUPPLEMENTARY MATERIAL

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