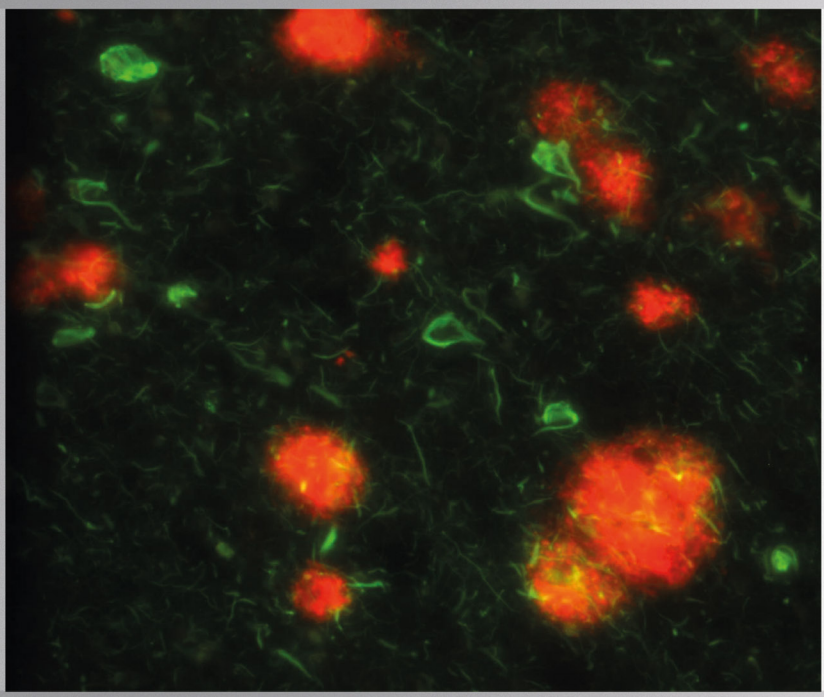
ADVANCES IN ALZHEIMER'S DISEASE 5

Handbook of Infection and Alzheimer's Disease



Edited by
Judith Miklossy

IOS Press

HANDBOOK OF INFECTION AND ALZHEIMER'S DISEASE

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.

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Handbook of Infection and Alzheimer's Disease

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This book is dedicated to all those who are facing Alzheimer's disease in the hope that it will contribute to open new opportunities for ground breaking health-care solutions to prevent this devastating disorder.

"A scientist who is also a human being cannot rest, while knowledge, which might reduce suffering rests on the shelf." - Dr Albert B. Sabin

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Preface

Every four seconds a new case of dementia is diagnosed. Never have people led healthier and longer lives. At a price: this has resulted in a tremendous increase in the number of people with dementia, as dementia mainly affects people when they get very old. But dementia is not a normal part of aging. The total number of people affected increases rapidly and might reach 120 million in 2050. Most of the affected people will be living in Asia, Latin America, or Africa. According to the World Health Organization (WHO), caring for and treating people with dementia currently costs the world more than 600 billion dollars per year.

Alzheimer's disease is the most frequent cause of dementia and has become a major public health concern; in fact the WHO declared dementia a public health priority.

As a previous State Secretary of Research and Education in Switzerland, I am aware of the challenge to governments and health systems, but also to families to care for the growing number of patients suffering from Alzheimer's disease. Finding a cure for this devastating disease is top priority. Many research organizations, thousands of research laboratories and hospitals, various biotech and pharma companies, patient organizations, and foundations have devoted enormous efforts to find therapeutic agents to cure or prevent this terrible disease, with few and disappointing results. Neurodegenerative diseases are so daunting that the most important—and rare—ingredient today is courage to follow new ideas, untrodden paths, and disruptive innovations.

As a trained neurologist, psychiatrist, and neuropathologist, Dr. Judith Miklossy has early on detected that spirochetes (helically shaped bacteria) when they invade brain cells, reproduce the filamentous pathology characteristic of Alzheimer's disease. Dr. Judith Miklossy has defended nearly single-handedly the hypothesis that chronic infection by spirochetes and several other important pathogens could constitute risk factors for Alzheimer's disease.

This is still a debated and largely underfunded alley of research in Alzheimer's disease. But if this hypothesis holds true, early intervention against infection may delay or even prevent the future development of Alzheimer's disease.

As a former State Secretary responsible for science policy, I always wondered how we could foster disruptive, innovative research. Granting agencies, with a well-established (and necessary) peer-review cycle, have trouble funding research for new disruptive ideas. Personalities like Judith, and foundations like the Prevention Alzheimer International Foundation established in Switzerland are the engines of this necessary type of research. Alzheimer's disease is too important and tragic for us to neglect any promising avenue.

Charles Kleiber PhD
State Secretary for Education and Research (1997-2008), Switzerland

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Introduction

There is a lack of awareness and understanding of dementia, which is often considered to be a normal part of ageing or a condition for which nothing can be done.

Dementia is declared to be a public health priority by the World Health Organization (WHO). Alzheimer's disease, which is the most frequent cause of dementia, is indeed an emerging public health problem. Following the estimation of WHO, the total number of patients with dementia worldwide is about 35.6 million and is expected to nearly double every 20 years, to reach around 115.4 million in 2050. The total estimated worldwide cost of dementia is around 604 billion per year.

If the cause of Alzheimer's disease is not defined, and treatments to delay or prevent the disease are not provided, the world will face an unprecedented health-care problem by the middle of this century.

Alzheimer's disease is the most frequent cause of dementia. The early clinical manifestations are subtle short-term memory deficits and anxio-depressive syndrome. During the slow progression of the disease, the intellectual functions progressively disappear and the patients survive in this devastating state of a complete dependence for more than a decade. The duration of the disease from the appearance of the first symptoms until the manifestation of dementia varies between 5 and 20 years.

The most characteristic pathological hallmarks of Alzheimer's disease are the accumulation of senile plaques and neurofibrillary tangles and the deposition of beta amyloid and pathologically phosphorylated tau protein in the affected brain. For the definite diagnosis of Alzheimer's disease the histological confirmation of these characteristic pathological changes are necessary.

All efforts made in research during the last four decades provided important insights into the pathogenesis of Alzheimer's disease but the cause of the disease is still unclear and the treatment unresolved.

A microbial origin of various chronic inflammatory disorders, including several neurodegenerative, neuropsychiatric and other systemic disorders is strongly intensifying. Accumulating historic and new observations provide evidence of an association between Alzheimer's disease and infectious agents, and open new opportunities to prevent dementia. Escaping host immune defenses microorganisms are able to initiate and sustain chronic infection and reproduce the pathological and biological hallmarks of Alzheimer's disease.

This handbook assembling and connecting findings with respect to the infectious origin of Alzheimer's disease is a first attempt to show that the accumulated data provided by hundreds of authors are worth of interest. The amount and the quality of data deserve the attention of the neuroscience community, physicians and health care authorities of governments worldwide. This approach brings new solution to define the cause of Alzheimer's disease and offer the possibility of a targeted therapy to prevent the disease. It is critical to take the right decisions today and not wait another century for the long awaited cure because the patients are continuously suffering.

The urgent need to explore the role of infectious agents in Alzheimer's disease is expressed by scientists around the world in the first chapter of the book.

That chronic inflammation is an important factor in the pathogenesis of Alzheimer's disease is well established and is summarized in the book by the pioneers of this important field of Alzheimer research.

Recent research demonstrated the involvement of various bacteria in Alzheimer's disease. *Chlamydia pneumoniae*, various spirochetes, including *Borrelia burgdorferi*, and periodontal pathogens, comprising *Porphyromonas gingivalis* and several periodontal pathogen spirochetes were detected in the brain in Alzheimer's disease, as reviewed in several chapters of the book. Biofilm formation of spirochetes in senile plaques and the association of polymicrobial periodontal disorders with Alzheimer's disease are also discussed. The bacterial burden and meta-analysis of bacterial infections in Alzheimer's disease are also highlighted in two distinct chapters.

Viral infections associated with Alzheimer's disease, including Herpes simplex virus type 1 (HSV1) and the anti-viral properties of β-amyloid peptides are also emphasized. Fungal infections occurring in AD is also revised.

The important role of bacterial amyloid as reviewed by one of the best experts in the field, the dysfunction of the blood-brain barrier, the role of iron, the long neglected important role of homocysteine as reviewed by its discoverer, the influence of genetic factors, including the role of ApoE4 genotype, and various environmental factors, with respect to the invasion

of the brain by pathogens in Alzheimer's disease, are the subjects of several chapters, which were contributed by pioneer scientists with international notoriety. The role of bacterial lipopolysaccharide (LPS) and the influence of pathogen free conditions on neurodegeneration in a mouse model of Alzheimer's disease are also discussed.

An infectious origin of Alzheimer's disease raises many important questions, which need an answer. One of them is related to the polymicrobial involvement of chronic inflammatory disorders. The association of various microorganisms with atherosclerosis, diabetes, several neurodegenerative disorders, including Alzheimer's disease, undoubtedly occurs. Nevertheless, the clinical, pathological and biological hallmarks of these disorders are distinct. Therefore, to define which microorganisms are able to reproduce the clinical and pathological hallmarks of a given disorder is important as it may influence the diagnosis, treatment and prevention strategies.

This book is the symbol of the effort and courage of all those who contributed to this new emerging field of Alzheimer's research, whether they participated in this book or not. I am grateful for all contributors of this book for their excellent work, for their generosity and their friendly and enthusiastic collaboration. I fully appreciate the help of the editorial board of the Journal of Alzheimer's Disease (JAD). Particularly I thank Beth Kumar, Managing Editor of JAD, for her prompt help any time I needed it. I am indebted to Rasjel van der Holst, Associate publisher of JAD, IOS Press, for her interactive, kind, and efficient collaboration that I fully appreciated. They both strongly contributed to the realization of this book. I am also very grateful for all those who participated in the time-consuming review process of the chapters in order to provide an objective and high quality book.

It was a pleasure to collaborate with all those who participated in the realization of this book and to give a helping hand to edit this book on the infectious origin of Alzheimer's disease, which is based on articles and reviews published in JAD during the last ten years. It is the open-minded approach of the journal, which enabled us to realize this book, which will certainly guide future generation to follow a path, which has been suggested by Oskar Fischer more than a century ago.

The final merit of the realization of this book is due to George Perry, the Editor in Chief of JAD and serial Editor of JAD Handbooks, who initiated and supervised the project, and helped in an accurate, diplomatic and scientific way during all the editorial process. I would like also to remember of Mark Smith previous co-editor of JAD, who I had the chance to know and witness the same open minded approach and courage with respect to this new line of Alzheimer's research.

It is expected that in the future many other books will appear on this important topic of Alzheimer's research. Important results are emerging, e.g. from Massachusetts General Hospital and Harvard University, showing that amyloid beta is an antimicrobial peptide, which accumulates in the Alzheimer's brain in response to invading pathogens. The courage of Robert Moir and Rudolf Tanzi to redirect genetic research to investigate an infectious origin of Alzheimer's disease is noteworthy. Drexel University also joined this new line of research highlighting the spirochetal origin and biofilm nature of senile plaques and contributing with a chapter to this book. There are many other ongoing research related to this new topic around the world with the participation of many other universities.

We hope that this first book, will contribute to a breakthrough in the history of Alzheimer's disease showing the right way to solve one of the most devastating disorders of the human being. As predicted by Thomas Lewis, we hope that to follow this path will conduct us to the "End of Alzheimer's disease" – as stated in the title of his book.

As so nicely expressed by Katherine Bick and Luigi Amaducci in the introductory remarks of their book on Alzheimer's disease, "it may be our generation's good fortune to reach the high ground and see answers plainly. Such is our goal."

Judith Miklossy

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Section 1
A neglected side of Alzheimer's research

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Infection and Alzheimer's Disease
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Microbes and Alzheimer's Disease

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We are researchers and clinicians working on Alzheimer's disease (AD) or related topics, and we write to express our concern that one particular aspect of the disease has been neglected, even though treatment based on it might slow or arrest AD progression. We refer to the many studies, mainly on humans, implicating specific microbes in the elderly brain, notably herpes simplex virus type 1 (HSV1), *Chlamydia pneumoniae*, and several types of spirochaete, in the etiology of AD [1–4]. Fungal infection of AD brain [5, 6] has also been described, as well as abnormal microbiota in AD patient blood [7]. The first observations of HSV1 in AD brain were reported almost three decades ago [8]. The ever-increasing number of these studies (now about 100 on HSV1 alone) warrants re-evaluation of the infection and AD concept.

AD is associated with neuronal loss and progressive synaptic dysfunction, accompanied by the deposition of amyloid-β (Aβ) peptide, a cleavage product of the amyloid-β protein precursor (AβPP), and abnormal forms of tau protein, markers that have been used as diagnostic criteria for the disease [9, 10]. These constitute the hallmarks of AD, but whether they are causes of AD or consequences is unknown. We suggest that these are indicators of an infectious etiology. In the case of AD, it is often not realized that microbes can cause chronic as well as acute diseases; that some microbes can remain latent in the body with the potential for reactivation, the effects of which might occur years after initial infection; and that people can be infected but not necessarily affected, such that 'controls', even if infected, are asymptomatic [2].

EVIDENCE FOR AN INFECTIOUS/IMMUNE COMPONENT

(i) Viruses and other microbes are present in the brain of most elderly people [11–13]. Although usually dormant, reactivation can occur after stress and immunosuppression; for example, HSV1 DNA is amplified in the brain of immunosuppressed patients [14].
(ii) Herpes simplex encephalitis (HSE) produces damage in localized regions of the CNS related to the limbic system, which are associated with memory, cognitive and affective processes [15], as well as personality (the same as those affected in AD).
(iii) In brain of AD patients, pathogen signatures (e.g., HSV1 DNA) specifically colocalize with AD pathology [13, 16, 17].
(iv) HSV infection, as revealed by seropositivity, is significantly associated with development of AD [18–21].
(v) AD has long been known to have a prominent inflammatory component characteristic of infection (reviewed in [22, 23]).
(vi) Polymorphisms in the apolipoprotein E gene, *APOE*, that modulate immune function and susceptibility to infectious disease [24], also govern AD risk (reviewed in [25, 26]).

Genome-wide association studies reveal that other immune system components, including virus receptor genes, are further AD risk factors [27–32].

(vii) Features of AD pathology are transmissible by inoculation of AD brain to primates [33, 34] and mice [35, 36].

EVIDENCE FOR CAUSATION

(i) In humans, brain infection (e.g., by HIV, herpesvirus, measles) is known to be associated with AD-like pathology [37–42]. Historical evidence shows that the clinical and pathological hallmarks of AD occur also in syphilitic dementia, caused by a spirochaete [4].

(ii) In mice and in cell culture, Aβ deposition and tau abnormalities typical of AD are observed after infection with HSV1 [43–52] or bacteria [16, 53–55]; a direct interaction between AβPP and HSV1 has been reported [56]. Antivirals, including acyclovir, *in vitro* block HSV1-induced Aβ and tau pathology [57].

(iii) Olfactory dysfunction is an early symptom of AD [58]. The olfactory nerve, which leads to the lateral entorhinal cortex, the initial site from where characteristic AD pathology subsequently spreads through the brain [59, 60], is a likely portal of entry of HSV1 [61] and other viruses [62], as well as *Chlamydia pneumoniae*, into the brain [63], implicating such agents in damage to this region. Further, brainstem areas that harbor latent HSV directly irrigate these brain regions: brainstem virus reactivation would thus disrupt the same tissues as those affected in AD [64].

GROWING EVIDENCE FOR MECHANISM: ROLE OF Aβ

(i) The gene encoding cholesterol 25-hydroxylase (CH25H) is selectively upregulated by virus infection, and its enzymatic product (25-hydroxycholesterol, 25OHC) induces innate antiviral immunity [65, 66].

(ii) Polymorphisms in human *CH25H* govern both AD susceptibility and Aβ deposition [67], arguing that Aβ induction is likely to be among the targets of 25OHC, providing a potential mechanistic link between infection and Aβ production [68].

(iii) Aβ is an antimicrobial peptide with potent activity against multiple bacteria and yeast [69]. Aβ also has antiviral activity [70–72].

(iv) Another antimicrobial peptide (β-defensin 1) is upregulated in AD brain [73].

Regarding HSV1, about 100 publications by many groups indicate directly or indirectly that this virus is a major factor in the disease. They include studies suggesting that the virus confers risk of the disease when present in brain of carriers of the ε4 allele of *APOE* [74], an established susceptibility factor for AD (*APOE ε4* determines susceptibility in several disorders of infectious origin [75], including herpes labialis, caused usually by HSV1). The only opposing reports, two not detecting HSV1 DNA in elderly brains and another not finding an HSV1–*APOE* association, were published over a decade ago [76–78]. However, despite all the supportive evidence, the topic is often dismissed as 'controversial'. One recalls the widespread opposition initially to data showing that viruses cause some types of cancer, and that a bacterium causes stomach ulcers.

In summary, we propose that infectious agents, including HSV1, *Chlamydia pneumonia*, and spirochetes, reach the CNS and remain there in latent form. These agents can undergo reactivation in the brain during aging, as the immune system declines, and during different types of stress (which similarly reactivate HSV1 in the periphery). The consequent neuronal damage—caused by direct viral action and by virus-induced inflammation—occurs recurrently, leading to (or acting as a cofactor for) progressive synaptic dysfunction, neuronal loss, and ultimately AD. Such damage includes the induction of Aβ which, initially, appears to be only a defense mechanism.

AD causes great emotional and physical harm to sufferers and their carers, as well as having enormously damaging economic consequences. Given the failure of the 413 trials of other types of therapy for AD carried out in the period 2002–2012 [79], antiviral/antimicrobial treatment of AD patients, notably those who are *APOE ε4* carriers, could rectify the 'no drug works' impasse. We propose that further research on the role of infectious agents in AD causation, including prospective trials of antimicrobial therapy, is now justified.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/16-0152).

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Section 2
Inflammation and Alzheimer's disease

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Infection and Alzheimer's Disease
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Inflammation, Anti-inflammatory Agents, and Alzheimer's Disease: The Last 22 Years

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Abstract. Two basic discoveries spurred research into inflammation as a driving force in the pathogenesis of Alzheimer's disease (AD). The first was the identification of activated microglia in association with the lesions. The second was the discovery that rheumatoid arthritics, who regularly consume anti-inflammatory agents, were relatively spared from the disease. These findings led to an exploration of the inflammatory pathways that were involved in AD pathogenesis. A pivotal advance was the discovery that amyloid-β protein (Aβ) activated the complement system. This focused attention on anti-inflammatories as blockers of complement activation. More than 15 epidemiological studies have since showed a sparing effect of non-steroidal anti-inflammatory drugs (NSAIDs) in AD. A consistent finding has been that the longer the NSAIDs were used prior to clinical diagnosis, the greater the sparing effect. The reason has since emerged from studies of biomarkers such as amyloid-β (Aβ) levels in the cerebrospinal fluid and Aβ deposits in brain. They have established that the onset of AD commences at least a decade before cognitive decline permits clinical diagnosis. Such biomarker studies have revealed that a huge window of opportunity exists when application of NSAIDs, other anti-inflammatory agents, or complement activation blockers, could arrest further progress of AD, thus eliminating its manifestation. It can be anticipated that this principle will apply to many other chronic neurodegenerative diseases. Neuroinflammation, discovered in AD more than 30 years ago, has now become a major field of brain research today. Inhibiting it may be the key to successful treatment of many chronic neurological disorders.

Keywords: Biomarkers, complement, immunohistochemistry, membrane attack complex, NSAID, reactive microglia

INTRODUCTION

Two basic discoveries led to the development of neuroinflammation as a major field of brain research. The first was the immunohistochemical demonstration of reactive microglia in Alzheimer's disease (AD) brains [1, 2], and the second was that persons suffering from rheumatoid arthritis had a greatly reduced risk of developing AD [3]. The first was interpreted as indicating the existence of a chronic inflammation in AD brain, while the second was considered to be a beneficial consequence after use of non-steroidal anti-inflammatory drugs (NSAIDs) prior to clinical disease manifestation [3]. In the ensuing years, there have been over 4,000 reports expanding the evidence of chronic inflammation in AD brain. There are now a large number of reviews on the subject (e.g., [4]). Reactive microglia have been shown to produce free radicals and other neurotoxic substances that kill neurons in culture [5, 6]. Some activated T cells are also found in the brain parenchyma in AD [7–9]. Such cells release inflammatory mediators, including the powerful proinflammatory stimulants interleukin (IL)-1, IL6, tumor necrosis factor alpha (TNF- α), and

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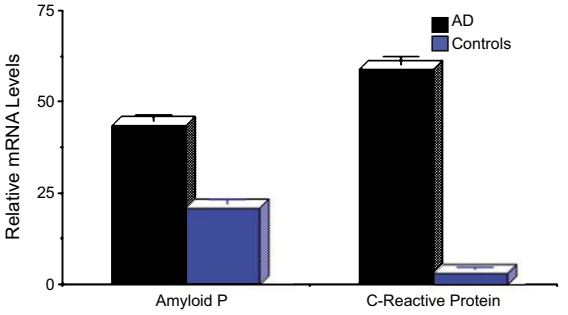

Fig. 1. Relative mRNA levels for amyloid P and C-reactive protein in the hippocampus of AD and control brain (data from [36]).

γ-interferon. Targeting TNF has been considered as a therapeutic strategy for AD [10].

A key discovery linking inflammation to AD was the finding that aggregated amyloid-β (Aβ) alone was a powerful activator of complement [11]. It had previously been shown that the complement system was activated in AD [12], but antibodies, which were then considered to be the main activators of complement, could not be identified in association with AD lesions.

The complement system has now been shown to be fully activated in AD [13]. Once activated, it produces anaphylatoxins, which promote further inflammation. Its opsonizing components mark material for phagocytosis. If fully activated, the membrane attack complex (MAC) is directly lytic to cells (Fig. 2). The MAC inserts itself into viable cell membranes, causing them to leak with subsequent death. It is intended to destroy foreign cells and viruses, but host cells are at significant risk in a phenomenon known as bystander lysis.

Figure 2 illustrates the difference between opsonization by the early complement components and the lytic effects of the MAC. Figure 2a shows double immunostaining for C4d, a fragment of the opsonizing pathway of complement, and complement receptors. C4d attaches covalently to the amyloid deposit, while activated microglia, which express high levels of complement receptors, are attacking the deposit by attraction to their ligand. Figure 2b shows a different phenomenon that is invisible to the stains used in Fig. 2a. This is the attack on neurites within the plaque by the terminal complement components C5b-9, which require a viable membrane for MAC assembly. The MAC has a very short half-life so finding immunohistochemical evidence for its existence in postmortem AD brains indicates the vigor of the attack. A more revealing overall index may be the levels of RNA expression of the complement proteins. There is a marked upregulation in affected regions in AD [14] (Fig. 3).

Identification of the MAC attacking dystrophic neurites in AD [13, 15] provides strong evidence of self attack in AD. It also provides the only *in vivo* evidence linking Aβ deposits with neurotoxicity. A host of other inflammatory markers have now also been shown to be upregulated in affected brain areas in AD. They include many of the inflammatory cytokines and such inflammatory stimulants as ICAM-1. Alleles that favor production of IL-1α, IL-1β, and IL-8, as well as TNF and other inflammatory cytokines, have been frequently reported to increase the risk of AD [16].

In the past 25 years, there have been more than 15 epidemiological studies showing that individuals are relatively spared from AD if they have been taking NSAIDs, or have suffered from conditions where such drugs are routinely used [4, 10, 17–21]. Four large epidemiological studies have analyzed

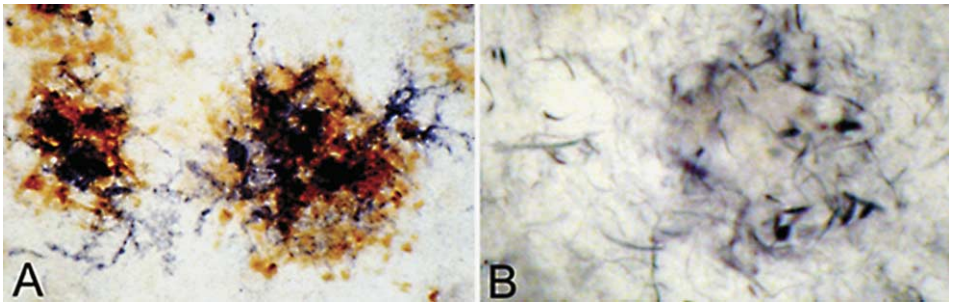

Fig. 2. Contrast in the immunostaining of AD senile plaques by opsonizing fragments of complement compared with the terminal components C5b-9. A) Double immunostaining for C4d and CD11c. The opsonizing fragment C4d (light brown) is covalently attached to plaque amyloid-β. Reactive microglia expressing high levels of the complement receptor CD11c (dark purple) agglomerate around the C4d ligand. B) Immunostaining for the MAC (C5b-9). Dystrophic neurites within the senile plaque are prominently stained, indicating lytic attack on trapped neuronal processes. Note the very weak immunostaining of neuropil threads in the surround.

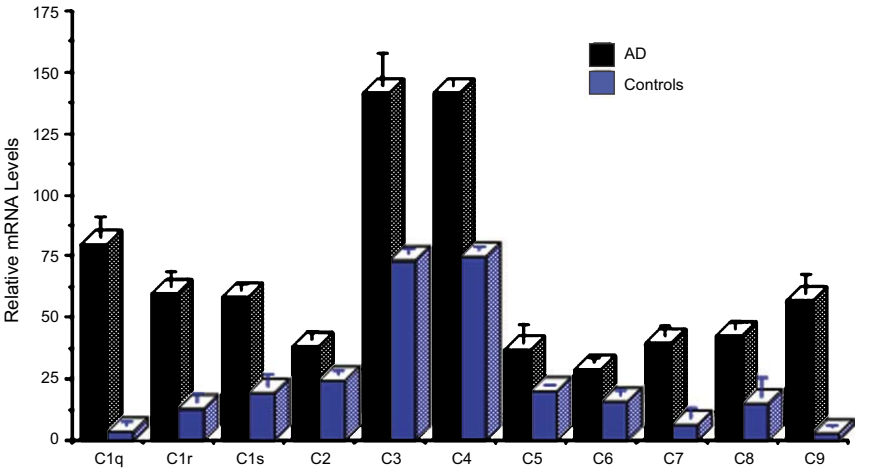

Fig. 3. Relative mRNA levels for proteins of the complement cascade in the hippocampus of AD and control brains (data from [37]).

the effects of NSAID consumption on AD. The Baltimore longitudinal study [18] showed a sparing of approximately 60% among NSAID users of greater than 2 years duration; the Cache County study showed a sparing of approximately 55% [21]; the Rotterdam study, where NSAID consumption was verified through prescription records, showed an 80% sparing [19]; and the MIRAGE study showed a sparing of 36% [20]. Some NSAIDs at very high doses directly bind to Aβ, but Szekely et al. showed they were no more effective than other NSAIDs in reducing the risk of AD [22].

BIOMARKER STUDIES

Biomarker studies have opened up a new era for AD research. So far there are three reliable types: cerebrospinal fluid (CSF) to determine Aβ and tau secretion levels, positron emission tomography (PET) to determine Aβ deposit levels (Pittsburgh Compound B, PIB), PET to determine metabolic rate (FDG), and MRI to determine brain volume. Overall, they show that AD onset occurs a decade or more before clinical symptoms appear.

Bateman et al. [23], in a landmark study of 128 participants, found that concentrations of $A\beta_{42}$ in the CSF declined 25 years before the expected clinical onset. Aβ deposits in the brain, as revealed by PIB, were detected 15 years before onset. They were concomitant with increased tau in the CSF and an increase in brain atrophy. Impaired episodic memory was observed ten years before the expected clinical diagnosis, and declines in the Mini-Mental State Examination and the Clinical Dementia Rating scale were detected 5 years before the expected clinical diagnosis.

Comparable findings were reported by Villemagne et al. [24], who estimated that it took 19.2 years of linear Aβ accumulation, 4.2 years of hippocampal atrophy, and 3.3 years of memory impairment to reach AD clinical diagnostic levels. Seppala et al. [25] correlated CSF findings with cortical biopsy analysis and found that patients with Aβ cortical plaques in biopsy samples had lower $A\beta_{42}$ CSF levels than those without plaques. Prestia et al. [26], following patients with minimal cognitive impairment (MCI), found that conversions to dementia increased as patients progressed from the appearance of $A\beta_{42}$ in the CSF, to abnormal $A\beta_{42}$ and FDG by PET, to hippocampal atrophy with $A\beta_{42}$ and FDG by PET.

Okonko et al. [27] found that abnormal $A\beta_{42}$ in the CSF, but not tau alterations, were associated with increased risk of AD, and Buchhave et al. [28] reported similar results in a study of patients with MCI followed for a median of 9.2 years. They concluded that 90% of patients with MCI and pathologic CSF biomarkers develop AD within 9 to 10 years, and that $A\beta_{42}$ is being deposited 5–10 years before the appearance of dementia. Shaw et al. [29] measured CSF biomarkers in mild AD and MCI patients compared with controls, as well as autopsy confirmed cases compared with controls. They concluded that $A\beta_{42}$ plus total tau predicted conversion of MCI to AD and that $A\beta_{42}$ was the most sensitive marker in the autopsy cases. Similarly, Visser et al. [30], in the DESCRIPA study involving a prospective

cohort, found that patients with an AD profile in their CSF were prone to advance from MCI to AD type dementia.

It is important to recognize that there is a fundamental difference between CSF and brain biomarkers. CSF turns over every 4–6 hours so CSF biomarkers provide a differential measure, revealing only the rate of production during that brief period of time. Brain biomarkers have a cumulative effect, so they are integral markers representing years of activity. Zetterberg et al. [31] found that CSF biomarker production remained constant over a two-year period as cognitive activity declined. Mattson et al. [4] found CSF biomarker production to be constant over a four-year period in MCI patients. Both these groups reached the conclusion that a CSF profile shifting toward normal would be useful in tracking disease-modifying drugs.

As for Aβ deposits in the brain, Vlassenko et al. [32] found that scans with PIB about 2 years apart in cognitively normal adults showed that those with elevated binding also showed enhancement of such binding, indicative of increased brain Aβ deposits. They concluded that a major growth in the Aβ burden occurs during a preclinical stage of AD. Bruck et al. [33] compared the prognostic value of PIB-PET, FDG-PET, and hippocampal volume MRI, for their prognostic value in predicting conversion of MCI to AD. Of the 29 patients, 17 converted to AD after 2 years. They concluded that the PET methods were superior to hippocampal volume methods in predicting the conversion. Hatashita and Yamasaki [34] followed 68 MCI patients by PIB-PET and FDG-PET. Over 19 months, 44% of the patients converted to AD. They found PIB-PET to be the most definitive marker of MCI. Jack et al. [35] have proposed a model in which Aβ biomarkers become abnormal first, with neurodegenerative biomarkers becoming abnormal later, correlating with clinical symptom severity. To apply these findings on a widespread basis to inhibit AD development, a simple, cheap, non-invasive test of disease onset is required.

CONCLUSIONS

In summary, biomarker data indicate that AD onset can be detected at least ten, and possibly 20 years prior to clinical diagnosis. They suggest that an extended window of opportunity exists for appropriate AD therapy to ameliorate, or even to prevent disease development. Such therapy would involve administration of NSAIDs and other anti-inflammatory agents. Widespread application will require development of a simple and reliable diagnostic method.

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Inflammasome Involvement in Alzheimer's Disease

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Abstract. Inflammasomes are responsible for the maturation of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-18, and IL-33 and activation of inflammatory cell death, pyroptosis. They assemble in response to cellular infection and stress or to tissue damage, promote inflammatory reactions, and are important in regulating innate immunity particularly by acting as platforms for activation of caspase proteases. They appear to be involved in several pathological processes activated by microbes including Alzheimer's disease (AD). Best characterized in microbial pathogenesis is the nucleotide-binding domain and leucine-rich repeat (NLR)-protein 3 (NLRP3) inflammasome. AD is a neurodegenerative condition in which the neuropathological hallmarks are the deposition of amyloid-β (Aβ) and hyperphosphorylated tau protein coated neurofibrillary tangles. For decades, the role of the innate immune system in the etiology of AD was considered less important, but the recently discovered inflammatory genes by genome-wide association studies driving inflammation in this disease has changed this view. Innate immune inflammatory activity in the AD brain can result from the pathological hallmark protein Aβ as well as from specific bacterial infections that tend to possess weak immunostimulatory responses for peripheral blood myeloid cell recruitment to the brain. The weak immunostimulatory activity is a consequence of their immune evasion strategies and survival. In this review we discuss the possibility that inflammasomes, particularly *via* the NLR family of proteins NLRP3 are involved in the pathogenesis of AD. In addition, we discuss the plausible contribution of specific bacteria playing a role in influencing the activity of the NLRP3 inflammasome to AD progression.

Keywords: Alzheimer's disease, amyloid-beta, bacteria, cytokines, inflammasome

INTRODUCTION

Inflammasomes are large intracellular multiprotein complexes that once activated *via* autoactivation or other biological triggers (bacterial RNAs) play a central role in the regulation of receptors and sensors of the innate immune system in relation to pyroptotic cell death [1–3]. Post activation of the inflammasome, caspase 1 enzyme initiates the maturation of pro-inflammatory cytokines particularly interleukin (IL)-1β, IL-18, and IL-33 [4] (Fig. 1), and inflammation mediated cell death occurs *via* the nucleotide-binding domain and leucine-rich repeat (NLR) family of proteins [5]. Inflammasomes assemble in response to various stimuli including cellular infection and stress or to tissue damage. They promote inflammatory reactions and are important in regulating innate immunity in chronic inflammatory diseases such as periodontitis and related systemic pathologies, for example, atherosclerosis, and metabolic and cognitive deficit diseases such as diabetes and dementia [6, 7].

A recent longitudinal study concluded that in AD, the presence of periodontitis was associated with a marked increase in cognitive decline over a six-month follow-up period, independent of the baseline cognitive state [8]. This and other similar longitudinal studies [9] have so far been unable to elucidate the true causation of AD development following periodontitis. The main hypothesis to explain the relationship between these two diseases is the age-dependent contribution of systemic inflammatory components

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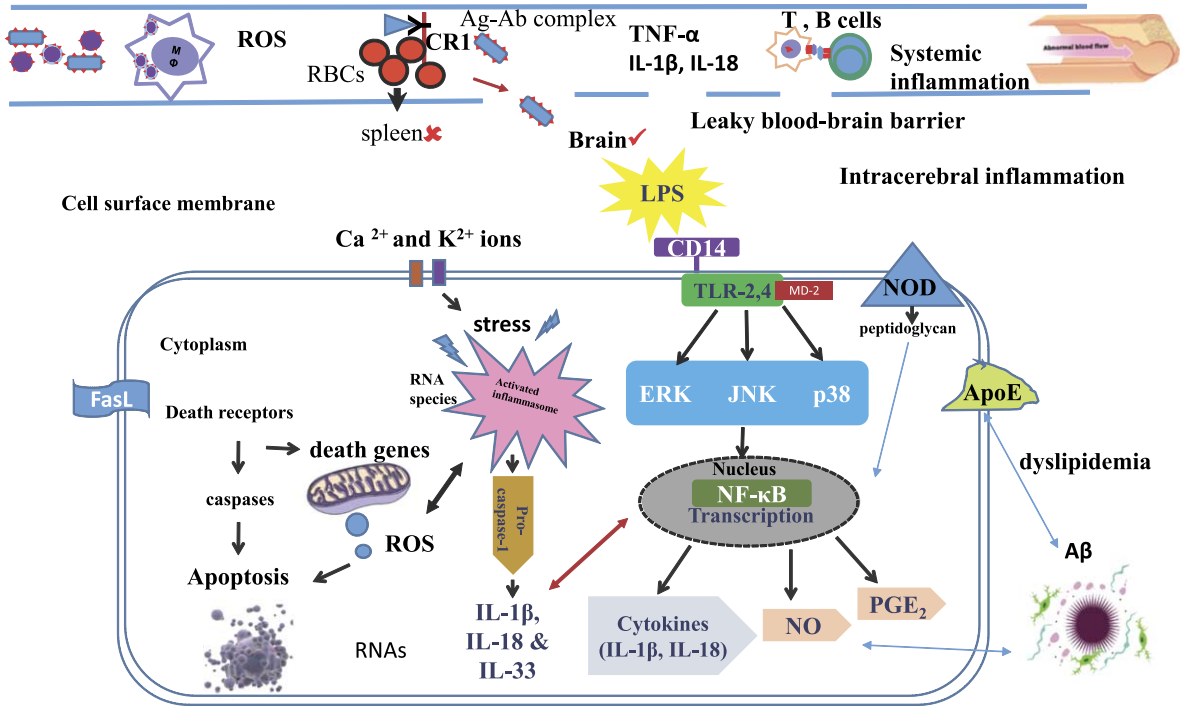

Fig. 1. Inflammasome activation in the brain with a leaky blood-brain barrier. Pathogenic PAMPs from bacteria "prime" the inflammasome *via* activating TLRs/NOD receptors that induce NF-κB activation and the expression of cytokines pro-IL-1β and IL-18. IL-33 secretion is mediated by both procaspase-1 and NF-κB. The inflammasome may recruit apoptosis-associated speck-like protein containing a carboxy-terminal CARD-like domain (ASC), and procaspase-1 in response to death activation signals. The inflammasome activation triggers include reactive oxygen species released from damaged mitochondria, stress, and exogenous RNA species. Once activated, the inflammasome causes the activation of caspase-1, which cleaves the precursor pro-forms of IL-1β, IL-18, and IL-33 into their mature forms. IL, interleukin; LRR, leucine-rich repeat; NACHT, central nucleotide-binding and oligomerization; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; TLR, Toll-like receptor; MD, MyD88 adapter protein; PGE2, prostaglandins; NO, nitric oxide; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinases; p38, p38 mitogen-activated protein kinase; ApoE, apolipoprotein E; NOD, nucleotide oligomerization domain; RBCs, red blood cells.

from periodontitis to the brain years before frank dementia develops [10–12]. We propose that the same hypothesis should now include the inflammasome involvement in the development of dementia *via* both extrinsic and intrinsic inflammatory mediator hypothesis of periodontal disease to explain the development of distant organ common inflammatory pathologies.

Inflammasomes detect and respond to a large range of pathogen-associated molecular patterns (PAMPs), including bacterial flagellin, peptidoglycan (*via* nod-like receptors) [4], and damage-associated molecular patterns (DAMPs), such as uric acid, cholesterol crystals, and misfolded proteins. Inflammasome activation inevitably results in the secretion of cytokines, which modulate innate immune responses to deal with infections and subsequent inflammation (Fig. 1). NLR-protein 3 (NLRP3) is the best characterized inflammasome and is important due to its involvement in microbial pathogenesis [13].

We have previously discussed how oral microbes can be involved in AD [14] and how the periodontopathogen *Porphyromonas gingivalis* (*P. gingivalis*) can modify the activity of the inflammasome [7]. In the present review, we discuss the possibility how inflammasomes, particularly the NLRP3 complex, may be involved in the pathogenesis of AD and that bacteria influence the activity of this inflammasome during AD pathogenesis.

THE NLRP3 INFLAMMASOME

An inflammasome is a multilateral macro molecule containing either pyrin or apoptosis-associated speck-like protein containing a carboxy-terminal CARD-like domain (ASC) and a central nucleotide binding domain (NACHT), and the C-terminus containing leucine-rich repeats that recognize pathogens and control autoregulation [15, 16]. Among the

inflammasomes detected, some are particularly well characterized for their role in bacterial recognition. These include NLR-CARD4 (NLRC4), NLRP3, and absent in melanoma 2 (AIM2) inflammasomes [7]. Of these, the NLRP3 inflammasome can be activated *via* bacterial RNA species [3]. The concept of inflammasome involvement in neurodegenerative diseases is being intensively investigated, where its end result appears to be liberation of the interleukin (IL) family of cytokines. This family serves several biological functions but if inappropriately secreted can lead to manifestation of depressive behaviors typically associated with dementia onset and chronic neuroinflammation. One of the roles being attributed to NLRP3 is its role in mitochondrial function impairment especially *via* oxidative stress responses, which link neurodegenerative disease conditions [17].

Unlike the peripheral immune surveillance by cells from the classical adaptive immune system derived from peripheral blood, the brain relies on its resident glial cells to provide a local innate defense mechanism capable of defending the CNS against pathogen entry. Most importantly, microglia are HLA-class II positive cells capable of antigen presentation as well as expressing CD14, toll like receptors (TLRs) 2 and 4 that recognize pathogen associated molecular patterns (PAMPs) and a range of complement receptors 1, 3, and 4 (CR1, CR3, and CR4) that identify and phagocytize bacteria [18] and extracellular amyloid-β (Aβ) of plaques.

Production of amyloid-β

The concept of AD resulting from initial infections, e.g., Lyme disease and general pareses, with eventual deposition of typical hallmark proteins (Aβ and tau protein coated tangles) [19, 20] would make excellent examples for inflammasome involvement in the production of Aβ. Acute phase proteins following bacterial infection would be stimulated by cytokines (IL-1, IL-6, IL-8, and TNF-α) and cytokines interact with inflammasome formation and activation. In addition, non-specific killing mechanisms that classically deal with bacterial infections and release of oxidative species following phagocytosis of bacteria by microglia and subsequently the complement system also maintain cytokine synthesis and liberation [21]. It is reported that bacterial infections that eventually result in AD also contribute to Aβ from Aβ-like proteins expressed on their surface membranes that cross react with the same antibodies that detect classical Aβ plaques of AD [20].

Complement activation in health facilitates clearance of foreign agents by coating microbes with immune complexes and opsonins (C1q, C3b, and iC3b). Additional activation products, the anaphylatoxins (C4a, C3a, C5a), promote vasodilatation and stimulate cellular immune responses via monocyte/macrophage cells [18]. Exposure of phagocytic cells to C3a/C5a anaphylatoxins stimulates synthesis of chemokines and proinflammatory cytokines and thereby maintains a high inflammation burden. It has also been suggested that these toxic initiation signals increase the propensity of the activation of inflammasomes, which further lead to the initiation of Aβ proteostasis *via* caspase cascades and inflammatory responses in AD [22].

Effect of Aβ on the inflammasome

In AD, microglial cells and astrocytes express NLRP3, which in turn can detect Aβ plaques and act by secreting caspase-1 to activate IL-1β and IL-18 [23–25]. IL-33 and its receptor ST2 have also been detected in AD around Aβ and in activated glial cells compared to brains from control cases [26]. The sequence of events is thought to lead to the establishment of an inflammatory cell environment around the plaques that theoretically downregulate the amyloid-β protein precursor (AβPP) breakdown product Aβ, but instead impairs the phagocytic signals in microglia [27].

Inflammation in the CNS can have both pathological and protective effects depending on the biological circumstances [28]. According to Salminen et al. [22], the innate immune system of the brain can recognize toxic Aβ oligomers and larger fibrils ($A\beta_{1\text{-}42}$) as danger signals and then activate the innate immune defenses. Endogenous danger signals (DAMPs; alarmins) and PAMPs also play a crucial role in the initiation of the immune responses [29]. Therefore, another pathway that can cause inflammasome activation involves extracellular ATP (ATPe) that is released by degenerating neurons [30–33] and acts as an independent endogenous danger signal.

The purinergic $P2X_7$ receptor is a trimeric ATP-gated cation channel found mainly, but not exclusively, on immune cells [34]. $P2X_7$ activation is followed by a number of downstream events, including release of pro-inflammatory mediators, cell death, and proliferation. Therefore, $P2X_7$ plays important roles in various inflammatory, immune, neurologic, and musculoskeletal disorders. $P2X_7$ expressed by microglial cells will also activate the

NLP3 inflammasome [30, 32] and the expression of $P2X_7$ is likely to be increased in AD brains [35]. $P2X_7$ was particularly upregulated around Aβ plaques in a mouse model of AD [36].

Purinergic receptors and pattern recognition receptors (PRRs) on immune cells do not only serve as initial sensors of microbial pathogens. They induce downstream inflammatory cascades associated with cognitive diseases such as AD and other major depressive disorders including Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis [37]. Pattern recognition receptors such as the TLR4 receptor are expressed in the brain's own immune cells like microglia and astrocytes that induce inflammation *via* cytokine secretion [38]. These receptors may also affect neurodegenerative diseases by inflammatory responses [39].

ACTIVATION AND INHIBITION OF CYTOKINES

As mentioned earlier, inflammasomes are engaged in the maturation of pro-inflammatory cytokines such as IL-1β, IL-18, and IL-33 [4, 40]. Overexpression of these interleukins is critical for the onset of the inflammatory processes that exacerbate pathology [41]. Increased levels of IL-1β and IL-18 have been detected in serum, cerebrospinal fluid, and brains of patients with AD and in other forms of dementia [42–46]. Also *in vitro* cell cultures of astrocytes express IL-18 constitutively, whereas the cytokine release is induced in microglia by bacterial lipopolysaccharide (LPS) [47]. It is noteworthy that IL-1β and IL-18 can activate various cell types, particularly astrocytes and microglia to induce additional cytokine release involving IL-1β, IL-6, and IL-18, and also nitric oxide (NO) synthase that can stimulate production of free radical NO, leading to the formation of peroxynitrite that denatures DNA and impairs cellular energy pathways [48, 49]. NO can also bring about apoptosis of hippocampal neurons *via* caspase-3 activity [50] whereas astrocyte-secreted IL-1β can increase the production of AβPP and Aβ from neurons [51–53] (Fig. 1). Additionally, it can induce phosphorylation of the tau protein and promote formation of neurofibrillary tangles through the mitogen activated protein kinases-p38 (MAPK-p38) stress pathway [22, 54].

Pro-inflammatory IL-18 increases AD-associated Aβ deposition in human neuron-like cells in culture [55]. IL-18 also increases the expression of glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase 5, both of which are involved in hyperphosphorylation of the tau protein [56]. If intracerebral contribution of IL-1 β and IL-18 is insufficient, then the hippocampus is also prone to a leaky blood-brain barrier (BBB) during aging [57]. This implies that the vulnerable risk age for onset of the late-onset AD is likely to suffer from micro bleeds and with it will enter the associated peripheral inflammatory mediators (Fig. 1). It is therefore not surprising to note that a significant increase in IL-18 detected in stimulated mononuclear cells and macrophages of peripheral blood from AD patients [58, 59] can be a contribution from the leaky BBB and would be expected to contribute to the intracerebral AD inflammasome. Noteworthy, IL-18 gene polymorphisms can predict risk and outcome of AD, suggesting that IL-18-mediated immune mechanisms can have an important role in AD pathogenesis [60].

There was no significant upregulation of IL-18 in severe AD patients compared to age-matched controls, whereas mild AD patients showed a significant increase in IL-18 [61], suggesting as AD progresses, the balance in the cytokine profile also changes. Accordingly, there is a gradual decline in the immune response in AD patients that might imply that IL-18 is an initiator of AD rather than an end stage mediator of continued neurodegeneration [62]. Both these studies indicated an important role of IL-18 in AD.

Both IL-1β and IL-18 are generated in their mature secreted form by caspase-1 through activation of the inflammasome. *Borrelia burgdorferi* (*B. burgdorferi*) infections associated with AD activate the nucleotide oligomerization domain-2 (NOD-2) pathway in microglia, ultimately leading to the secretion of inflammatory cytokines that directly target oligodendrocytes and neurons for apoptotic cell death resulting in axonal degeneration [62]. However, IL-18 can be derived as a byproduct from the activities of various extracellular enzymes such as protease 3, serine protease, elastase and cathepsin G [62–65]. Interestingly, IL-1β and IL-18 can be regulated by the same inflammasome or by different inflammasomes. Thus IL-1β and IL-18 are secreted from primed murine dendritic cells in response to *Listeria* protein p60, but inhibition of NLRP3 reduced the production of IL-1β but not IL-18 [66]. Therefore, maturation of IL-1β and IL-18 could be regulated conditionally by different signaling mechanisms [62]. Unfortunately, there is currently no evidence to suggest that completely blocking IL-1β or IL-18 will improve the human form of AD [67]; however, our own opinion is

that a dampened response of the cytokines at an early stage of the disease process may be beneficial.

ACTIVATION AND INHIBITION OF CASPASE-1

In the NLRP3 inflammasome, the NLR protein recruits the inflammasome-adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), which in turn interacts with caspase-1 leading to its activation [7]. Once activated, caspase-1 promotes the maturation of the proinflammatory cytokines IL-1β, IL-18, and IL-33. The NLRP3 inflammasome has a role in AD by increasing caspase-1 expression levels in AD brains [13, 23]. Knockout of NLRP3 and caspase-1 suppressed amyloidogenesis and neuropathology and improved cognition in AD transgenic mice [23]. By exposing LPS-primed macrophages to fibrillary Aβ, caspase-1 was activated and IL-1β release triggered [68]. The response depended on NLRP3 and involved both endosomal rupture and cathepsin B release. Heneka et al. [23] reported a strongly enhanced caspase-1 expression in human mild cognitive impairment and brains with AD suggesting a role for the inflammasome in this neurodegenerative disease. Active caspase-6 and caspase-6-cleaved γ-protein were detected in neuropil threads, neuritic plaques, and neurofibrillary tangles in AD [69]. This prompted Salminen et al. [22] to propose that the functional link between caspase-1 and caspase-6 connects the activation of inflammasomes to apoptotic cell death and AD pathology.

BACTERIA REGULATE INFLAMMASOME ACTIVITY

As mentioned previously, inflammasomes detect and respond to a large range of PAMPs. After infection or cellular stress, inflammasomes are assembled, activated, and involved in the host defense and pathophysiology of the disease [70]. Infectious agents have been linked to cognitive decline in several reports [71–74] and surprisingly all of these microbes, including *P. gingivalis* [75] and *Treponema denticola* (*T. denticola*) [76] being highly inflammophilic, do not appear to be potent activators of myeloid cells in the brain. Metabolically active *P. gingivalis* has been shown to gain entry to the brain from its primary gingival location [77]. However, since proteinase gingipains (found in association with the outer surface membrane of *P. gingivalis*) would have been present, its significance may lie in the breakdown of vascular structures and cerebral parenchymal connective tissues strengthening their plausible association with eventual AD neuropathology and progressive BBB deterioration. Also studies with gene-deficient mice and cells have indicated that NLR inflammasomes are implicated in the host response of a wide range of microbial pathogens, inflammatory diseases, cancer, and metabolic and autoimmune disorders [78].

Inflammasomes have a highly adaptable scaffold suited for detecting and initiating rapid innate responses to diverse bacteria [79]. Thus the NLRP3 inflammasome sensing *Streptococcus pneumoniae* (*S. pneumoniae*) had a protective effect since mice deficient in NLRP3 had a more severe course of lung infection [80]. However, in a mouse model of *S. pneumoniae* meningitis NLRP3 inflammasome induction and the subsequent cytokine response increased brain pathology [81, 82]. IL-1β or IL-18 signaling had minimal impact on bacterial growth within the brain but promoted local, pathogen associated inflammatory responses [81, 82]. This is another example where inflammasome activation by bacteria was more harmful. The pneumolysin of *S. pneumoniae* induces IL-1β and TNF-α in human mononuclear cells possibly by a mechanism similar to other pore-forming toxins [78]. During infection of human dendritic cells secretion of IL-1β was increased indicating a dynamic role for pneumolysin in IL-1β maturation [83].

Microglial cells have a functional Naip5-NLRC4 inflammasome that is important in monitoring and clearing CNS infection from flagellated bacteria [84]. *Pseudomonas aeruginosa* (*P. aeruginosa*) is also a potent activator of the NLRC4 inflammasome. This is mediated by flagellin-dependent and –independent mechanisms [78]. Several pathogenic bacteria have developed strategies to counteract inflammasomes through "stealth" mechanisms [85]. One of these is *Staphylococcus aureus* (*S. aureus*) that can modify its cell wall peptidoglycan to prevent degradation by lysozymes through peptidoglycan O-acyl transferase A that strongly suppresses inflammasome activation and inflammation *in vitro* and *in vivo* [86]. Inhibition of the inflammasome has also been detected for *Yersinia* and *Mycobacterium* species [78]. *Yersinia* encodes a family of outer membrane proteins, Yops, that is injected into the cytosol by the type III secretion system (T3SS). Among these proteins, YopE, YopT, and YopK inhibit inflammasome activity [87,

88]. The BCG strain of *Mycobacterium tuberculosis* (*M. tuberculosis*) encodes a Zn^{2+} metalloprotease *Zmp1* and suppresses inflammasome function [89]. On the other hand, *Francisella tularensis* (*F. tularensis*) does not induce a substantial pro-inflammatory response. The live vaccine strain of *F. tularensis* encodes two loci, *ripA* and *mviN*, that inhibit inflammasome activation [90, 91]. MviN, which is a flippase, inhibits caspase-1 activation in an AIM2-dependent manner.

We have recently highlighted that *P. gingivalis* has several mechanisms of modulating innate immune responses [92] and one of these is *via* activation of the NLRP3 inflammasome, e.g., through suppressing activation by another dental biofilm bacterium, *Fusobacterium nucleatum* (*F. nucleatum*), by using its extracellularly secreted nucleoside diphosphate kinase homologue (NDK), the purine receptors $P2X_4$/$P2X_7$, its A2a adenosine receptor, phosphatidylserine, and under acylated LPS [7, 93]. Among them, ATP-/$P2X_7$-signaling has been associated with periodontitis and with the development of several systemic diseases related to periodontitis such as AD [7]. To what extent inflammasome modification by *P. gingivalis* also occurs in the brain is not known. Theoretically, *P. gingivalis* might attenuate the inflammasome for its own survival [7]. However, even though *P. gingivalis* inhibits an activation pathway that can kill the bacterium, this may not be the integral part of a general immune suppression strategy since *P. gingivalis* harnesses acute sustained inflammation that is relatively harmless to the bacterium [7, 92]. Accordingly, periodontal bacteria, especially *T. denticola*, may contribute to AD pathology involving mechanisms such as acute phase proteins, cytokines, and the complement cascade in which neurons would be attacked even if these bacteria modulate inflammasome activity at a much slower pace than *P. gingivalis* due to their slow replication cycle in the brain [94].

Longitudinal studies including that by Ide et al. [8] all agree that in AD, the presence of periodontitis appears to be associated with a marked increase in cognitive decline. If periodontitis is a true risk factor for developing AD, then assessment of any disease requires longitudinal monitoring of patients. In this respect, dental intervention appears effective in ameliorating inflammatory effectors originating from oral pathogens [95].

In conclusion, innate immune responses, particularly those activating inflammasomes, may contribute to the onset and progression of AD. Inflammasomes may represent a collective neuroinflammatory pathway being involved in stimulation of cytokines that give rise to Aβ release and symptoms of depression. Therefore, inflammasomes might be a culprit in the pathology of AD. However, the intricate mechanisms of inflammasome assembly and activation in the CNS are not yet fully understood; neither is the precise role of the NLRP3 inflammasome in AD. The fact that NLRP3 inhibition could protect memory loss and decrease Aβ deposition in an AD mouse model supports the notion that inflammasome inhibitors may be applicable as therapeutic agents; however, history suggests results from animal studies do not automatically provide the same outcome in man. Although microglia, astrocytes, and neurons express inflammasomes, little is known about how this diversity of cells affects the regulation of IL-1β signaling at the tissue level. Also, inflammasomes have recently been found to release other immune substances than just IL-1β, IL-18, and IL-33, such as prostaglandins and leukotrienes. In the complex interplay of factors involved in AD pathogenesis, the inflammatory activators of NLRP3 inflammasome/caspase-1 are important. It is clear that the NLRP3 inflammasome is involved in the innate immune response to Aβ deposition and/or in its clearance. Soluble oligomeric assemblies of the Aβ peptide (ADDLs) seem to be the more toxic Aβ species and potent danger signals to activate the inflammasome. Microorganisms are also important both as activators and modifiers of inflammasome action as demonstrated in animal models. It is clear that pathogenic bacteria have developed a plethora of strategies to inhibit inflammasome-mediated processing of IL-1β and IL-18. The key pathobiont, *P. gingivalis*, is no exception in this sense as it can modify inflammasome activity in several ways. The importance of this in the CNS is not clear. It should be emphasized that it is probably not in the "interest" of *P. gingivalis* to completely inhibit inflammasome activity since it requires maintenance of some inflammation to obtain nutrients for its growth such as hemin and peptides from tissue breakdown. Modification of inflammasome activity might allow more room for other virulence factors of *P. gingivalis* to contribute in AD, particularly complement inactivation followed by neural injury from LPS/gingipains.

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Inflammatory Aspects of Alzheimer Disease and Other Neurodegenerative Disorders

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Abstract. Alzheimer and a number of other neurodegenerative diseases are characterized by the presence of reactive microglia and reactive astrocytes in association with the lesions. The classic view that microglia exist primarily in either a resting or activated state needs to be broadened in view of recent results. Resting microglia are in constant activity sampling their surround. Activated microglia may be pro-inflammatory, releasing inflammatory cytokines and other inflammatory mediators, or anti-inflammatory, promoting the healing process. There is evidence that microglial phagocytosis is more powerful in the anti-inflammatory state. Activated astrocytes also have pro-inflammatory and anti-inflammatory properties. In the pro-inflammatory state they release inflammatory cytokines. In the anti-inflammatory state they release various growth factors. In AD and other neurodegenerative diseases, both microglia and astrocytes are in a pro-inflammatory state. From a therapeutic point of view it is desirable to find methods of tipping the balance towards an anti-inflammatory state for both types of glia.

Keywords: Amyloid-β protein, amyotrophic lateral sclerosis, astrocytes, microglia, neurotoxicity, Parkinson disease, synuclein

INTRODUCTION

Inflammation is a key component of an innate immune response. Innate immunity is a highly conserved system that protects the host from infections and injury in a relatively non-specific manner. A multitude of factors are involved in the overall response such as cytokines, the complement system, acute phase reactants and various cellular elements, which in concert mount a powerful reaction. While this system is an effective and potent response to acute challenges, it is imperative that it be tightly regulated over the longer term. Dysregulation and chronic activation can have detrimental effects for the host by extending focal damage into nearby healthy tissue, a process termed 'bystander damage'. Controlling and reducing the damaging properties of inflammation have proven to be useful in the therapy of a number of peripheral disorders such as atherosclerosis and rheumatoid arthritis.

Chronic inflammation has been implicated not only in diseases of the periphery, but also in the central nervous system in neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease and amyotrophic lateral sclerosis (ALS). Expression of inflammatory mediators has been demonstrated in affected brain regions [45,48]. Some pharmacological inhibitors and modulators of inflammation, such as nonsteroidal anti-inflammatory drugs (NSAIDs),have been shown to exert protective effects in several epidemiological studies as well as in animal models of neurodegenerative disease [36,49]. Additionally, it has been demonstrated that genetic polymorphisms for several inflammatory cytokines and their receptors modify the risk for some neurodegenerative disorders [3,7,11,66].

This review focuses on the involvement of those cells primarily involved in the central nervous system inflammatory process. These are microglia and astroglia. Special attention will be paid to the regulation of specific states in microglial activation as well as the potential involvement of astroglial cells in the inflammatory process. Also, recent reports on the balance

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between pro- and anti-inflammatory processes will be reviewed. Finally, consideration will be given to potential peripheral inflammatory biomarkers and novel therapeutic strategies directed at the immune system.

MICROGLIAL STATES OF ACTIVATION

Microglial cells are considered to be the immune effector cells in the CNS, belonging to the mononuclear phagocytic system [46]. They express proteins characteristic of professional phagocytes and immune cell members such as complement components and their receptors, major histocompatibility complex (MHC) glycoproteins, and scavenger receptors. Activated microglia become highly mobile, in order to migrate and phagocytose identified targets. Upon stimulation they release cytokines, chemokines and other toxic substances such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and, with species specificity, express inducible nitric oxide synthase (iNOS) [16,33, 90]. Powerful activation leads to generation, through the respiratory burst system, of toxic free radicals [32].

It has long been assumed that microglia exist in two basic states: resting and reactive. When microglia encounter invading microorganisms or debris, they convert to the activated state. After the offender is removed, microglia return to the resting state or may be sacrificed by undergoing apoptosis. If the initiating disturbance persists, microglia may become chronically activated. In this state, an excess of toxic factors may be continuously released, resulting in spread of damage to viable surrounding tissue.

More recent studies have shown that this simplified view may need to be modified in at least two ways (for reviews see [24,26]). Firstly, the term 'resting state' implies inactivity, but microglia in this state are in dynamic activity. Time-lapse imaging in vivo by two-photon microscopy in transgenic mice has demonstrated that the ramified morphology typical for these cells is not static at all [15,59]. 'Resting' microglia continuously extend and retract their processes over the area covered by their ramifications. In this way microglia 'patrol' their surroundings and can react quickly to disturbances. It is possible that microglia in this surveillance mode constantly repair micro damage and act in a neuroprotective fashion. In support of this idea, it has been shown that microglia release growth factors (such as insulin-like growth factor-1, IGF-1 [8]) and may alleviate neurotoxiciy by glutamate uptake [61, 73].

Secondly, qualitatively different states of activation may exist. They may be pro-inflammatory or anti-inflammatory. Attempts have been made to characterize these differing states as in the Th1 and Th2 T-cell responses. In analogy to Th1, the pro-inflammatory state has been termed the 'classical' microglial response. In analogy to Th2, the 'alternative' response is anti-inflammatory. It assists in tissue repair. The classic pro-inflammatory response can be triggered by such inducers as bacterial lipopolysaccharide (LPS) and results in expression and release of pro-inflammatory, neurotoxic factors. The potency of such activation, and the neuronal consequences, have been demonstrated by Qian and coworkers who, by administration systemically of a single dose of LPS, induced microglia to kill substantia nigra neurons in mice [64]. This microglial activation was sustained for several months but was blocked in transgenic mice lacking TNF-α receptors [65] or MAC1 receptors [60]. Aspects of pro- and anti-inflammatory activation states of microglia are summarized in Table 1.

Aβ TRIGGERS PRO-INFLAMMATORY REACTIONS

It is widely acknowledged that amyloid-β (Aβ), especially in aggregated or fibrillary forms, triggers pro-inflammatory reactions of microglia. When primary cultures of human microglia were treated with Aβ, there was upregulation of gene transcription for pro-inflammatory cyto- and chemokines (IL-1β, IL-8, MMP, CCL) [84]. Aggregated Aβ, like bacterial lipopolysaccharide, evoked a cytotoxic reaction of microglia cocultured with hippocampal brain slices. There was increased expression of Ii, CIITA, STAT1, and TNF-α mRNA accompanied by induction of neuronal death [8].

A particularly significant property of aggregated Aβ is that it is a powerful activator of human complement [69]. The N-terminal residues of the beta pleated structure bind to human C1q, thus initiating the complement cascade [86]. This property explains the intense labeling of AD senile plaques with the opsonizing components of complement, and the finding of nearby dystrophic neurites being attacked by the membrane attack complex (MAC) [86]. Mouse C1q binds much more weakly than human C1q to human Aβ, which accounts for the weak complement activation in AD transgenic mice and the absence of MAC associated with dystrophic neurites [87]. This difference is of profound

Table 1
Aspects of pro- and anti-inflammatory activation states of microglia

Pro-inflammatory (classical) state of microglia activation		
Stimulus	Response	Experimental system, reference
LPS	Neurotoxicity in substantia nigra (blocked by deficiency in TNF-α receptor, MAC1 receptor)	Intracerebral injection [60,64,65].
Fibrillar/aggregated Aβ	Upregulation: IL-1β, IL-8, MMP, CCL Downregulation: Aβ receptor, MSR-1, CD36, CD47	Cell culture [84]
Fibrillar/aggregated Aβ	Ii, CIITA, STAT1, TNF-α Neurotoxicity	Co-culture microglia on hippocampal slice [8]
$\alpha-$synuclein	TNF-α, IL-1β	Culture with conditioned medium [37]
TNF-α, IL-1β, IFN-γ	Attenuation of Aβ phagocytosis	Cell culture [38,39,88]
LPS and IFNγ	Reduced AβPP phagocytosis	Cell culture [83]
CCR2 deficiency	Impaired microglia accumulation and reduced Aβ phagocytosis	Transgenic mouse strain [19]
Combinations of LPS, IFN-γ and/or TNF-α	Neurotoxicity	Culture with conditioned medium [35]
Aβ	TNF-α, IL-1β, iNOS, ROS	Cell culture [16,33,90]
Anti-inflammatory (alternative) state of microglia activation		
Stimulus	Response	Experimental system, reference
IL-4	Increased oligodendrogenesis	Rodent model of EAE [9]
IL-4 IFN-γ (low dose)	Increased neurogenesis via IGF-1	Co-culture [10]
Neuron culture supernatant	Neurotrophic	Culture with conditioned medium [56]
IL-4	Reduces neurotoxicity	Culture with conditioned medium [35]
Apoptotic neurons (phosphatidylserine)	Upregulation: PGE-2, TGF-β, NGF Downregulation: TNF-α, NO	Co-culture [52]
TGF-β, IL-1β	Modulation and decrease of activity	Culture, transgenic mice [5,42,72]
IL-10	Reduced LPS-induced neurotoxicity Downregulation: TNF-α, NO, superoxide	Neuron-microglia co-culture [63]
	IGF-1	Co-culture microglia on hippocampal slice [8]
	Neuroprotection via glutamate uptake	Co-culture, acute nerve injury model [61,73]
Astrocyte conditioned medium	Downregulation: iNOS, ROS	Culture [51]
CCL2 (MCP-1) CXCL10 (IP-10)	Modulation of activity and migration	Multiple sclerosis [78]
Fractalkine (CX3CL1)	Inhibition of neurotoxicity	Co-culture [53], transgenic mice [12]
Endocannabinoid (CB2) ligands	Reduced neurotoxicity, Downregulation: TNF-α, IL-1β	Supernatant of microglia/macrophage culture [34]
PPARγ agonists	Downregulation of activity, microglial death	Asthma mouse model, cell culture, others [27, 31,40,89]

significance in Aβ immunization experiments. In transgenic mice, vaccination fails to induce bystander lysis, but in humans it may be the principal reason why the Aβ vaccination trial resulted in sterile meningitis and other damaging side effects in some individuals [23, 58].

In addition to Aβ, other misfolded proteins causing so-called proteinopathies can activate microglia cells. Yoshiyama and colleagues showed that in P301S tau transgenic mice, microglial activation coincided with synapse loss and impaired synaptic function [91]. This preceded neuronal loss and tangle development. Tau pathology was reduced in animals treated with immunosupressive drugs [91]. α-synuclein, another protein subject to misfolding, increases secretion of IL-1β and TNF-α from human microglial and human THP-1 cells through signaling mechanisms via the three major mitogen-activated protein (MAP) kinase pathways: p38 MAP kinase, extracellular regulated protein-serine kinase (ERK)1/2 and c-Jun-N-terminal kinase (JNK) [37]. This indicates that similar inflammatory processes take place in disorders involving misfolding of tau and alpha-synuclein. This suggests that fronto-temporal dementia with parkinsonism, progressive supranuclear palsy, multiple system atrophy and Lewy body dementia may be targets for drugs that are able to modify microglial behavior.

Aβ IMPAIRS PHAGOCYTOTIC CAPACITY OF MICROGLIA

A precipitating cause of AD may be impaired phagocytosis of Aβ by microglia. A chronic inflammatory milieu may reduce the phagocytic capacity of microglia, as demonstrated by in vitro experiments

where treatment with IL-1β, TNF-α or IFN-γ resulted in reduced Aβ phagocytosis [38,39,88]. Walker et al reported that when primary cultures of human microglia were treated with Aβ, gene transcription for pro-inflammatory cyto- and chemokines was up regulated (IL-1β, IL-8, MMP, CCL), while gene transcription for Aβ-binding receptors MSR-1, CD36, and CD47 was down regulated or unchanged [84]. They concluded that, although microglia reacted in a pro-inflammatory manner, there was a deficit in phagocytosis and removal of Aβ [84].

Amyloid-β protein precursor (AβPP) or Aβ deposition may push the microglial balance towards pro-inflammatory type activation. For example, AβPP expression in APPsw transgenic mice is altered in favor of such an immune response. Crawford and colleagues subjected APPsw mice and their wild type littermates to traumatic brain injury [14]. While microglia in wild type mice reacted predominantly with an upregulation of anti-inflammatory factors, APPsw mice increased expression of pro-inflammatory genes and others related to a pro-inflammatory immune response, resulting in cell death. It was suggested that a particular vulnerability to head injury may exist in the Alzheimer brain [14].

In addition, it has also been demonstrated that pro-inflammatory conditions (LPS and IFN-γ) reduce phagocytosis of AβPP in primary rat microglial cultures [83].

In humans it has been reported that microglia become senescent and deteriorate with age, possibly decreasing their phagocytotic competence and exacerbating damage by abnormal protein depositions in neurodegenerative disorders [76].

MICROGLIAL ACTIVATION ESSENTIAL FOR PHAGOCYTOSIS AND REPAIR

Microglial activation plays a crucial role in acute models of neurotoxicity. Turrin and Rivet showed that neurodegeneration caused by injections of the nitric oxide donor sodium nitroprusside was more severe in TNF-α deficient mice compared to wild type controls and IL-1β deficient mice. The initial microglial response in the TNF-α deficient mice was slowed down, but was followed by an exaggerated activation. It was suggested that early activation is necessary to eliminate cell debris, restrict subsequent damage, and restore homeostasis [80]. Injection of IL-4 activated microglia increased spinal cord oligodendrogenesis and improved the outcome in a rodent model of acute or chronic EAE [9]. Removal of apoptotic cellular debris appeared to trigger a Th2 like response, characterized by an anti-inflammatory and possibly neuroprotective state. This microglia-mediated clearance of debris seemed to be a prerequisite for repair in conditions like multiple sclerosis (MS) [9].

The importance of appropriate phagocytotic function of microglia for brain homeostasis has been demonstrated in Nasu-Hakola disease or polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL). This is a chronic neurodegenerative disease with dementia [57]. Loss-of-function mutations lead to a lack of triggering receptor expressed on myeloid cells-2 (TREM2). This impairs the phagocytotic capacity of microglia and increases the expression of pro-inflammatory cytokines.

In mouse models of AD, appropriate microglial functioning supports clearance of Aβ and lowering the plaque load. CCR2 is a chemokine receptor expressed on microglia which mediates the accumulation of mononuclear phagocytes at sites of inflammation [19]. It was shown that CCR2 is crucial for microglial clearance of Aβ in Tg2576 AβPP transgenic mice. CCR2 deficiency impairs microglial accumulation and causes a decrease in Aβ clearance in the early stages of pathology while accelerating accumulation of Aβ later on.

Co-culturing microglial cells with apoptotic, but not with healthy or necrotic neurons, induced release of the anti-inflammatory and neuroprotective substances PGE-2, TGF-β, and NGF while inhibiting release of the pro-inflammatory molecules TNF-α and NO, likely via phosphatidylserine-expression by the apoptotic neurons [52].

There is also a strong indication that certain forms of microglial activation aid Aβ removal in humans. This was shown in a case report of a patient with AD who suffered a stroke. The plaque load was reduced in the ischemic regions where highly activated microglia had accumulated [1]. As well, clearance of Aβ was found in selected patients in Elan's AN-1792 trial [58], but it is not clear if this was due to a specific antibody mediated response or to non-specific activation of microglia. Effects of immunization on microglia activation and Aβ clearance in transgenic mouse models are summarized in a review by Morgan [54].

For recent extensive reviews of the neuroprotective function of microglia see also [24–26].

Astroglial states of activation

Astrocytes are the most abundant glial cell type in the brain. Classically, their function has been considered to be that of supporting the structure of the brain and the metabolism of neurons. Astrocytes are also involved in formation and maintenance of the blood brain barrier and are essential for brain homeostasis. However, under pathophysiological conditions such as infection, injury, demyelinating and neurodegenerative disorders, astrocytes become activated with ensuing hypertrophy and proliferation. Reactive astroglia can form a glial scar in response to injury, leading to an isolation of injured tissue and accompanying inflammatory processes. More recently it has been shown that astroglia themselves actively contribute to the inflammatory response [18,22]. Astroglia can modify permeability of the BBB, aiding recruitment of immune cells from blood. They have been shown to express receptors involved in pathogen recognition and inflammation such as Toll-like receptors, as well as scavenger and complement receptors. They can also produce complement factors, complement inhibitors, chemokines, cytokines and neurotrophic factors (for review see [22]).

Astroglial cells support neuronal survival, regeneration and differentiation by expressing a wide range of growth factors. Among these are NGF, CNTF, BDNF and IGF-1, AFGF and BFGF [22]. The neuroprotective action of astrocytes has also been attributed to their capacity to take up the neurotransmitter glutamate, convert it to glutamine, and recycle it to neurons. But it has been shown that glutamate is released again from astrocytes in neuroinflammatory conditions and also to some degree under normal circumstances [82]. Pro-inflammatory cytokines, such as TNF-α, can stimulate this release via PGE-2 [6]. It has been shown that the TNF-alpha-triggered glutamate release is reduced in aged PDAPP transgenic mice, but when hippocampal slices are treated with PGE-2, glutamate release increases to control levels [70]. Blocking of either TNF-alpha or PGE-2 reduces glutamate release from astrocytes stimulated by purinergic P2Y1 receptor activation [17].

Astroglia in AD

While microglia infiltrate Aβ plaques in AD, astrocytes aggregate around the periphery, walling off the area. This positions three key players: Aβ, microglia, and astroglia in close proximity (Fig. 1).

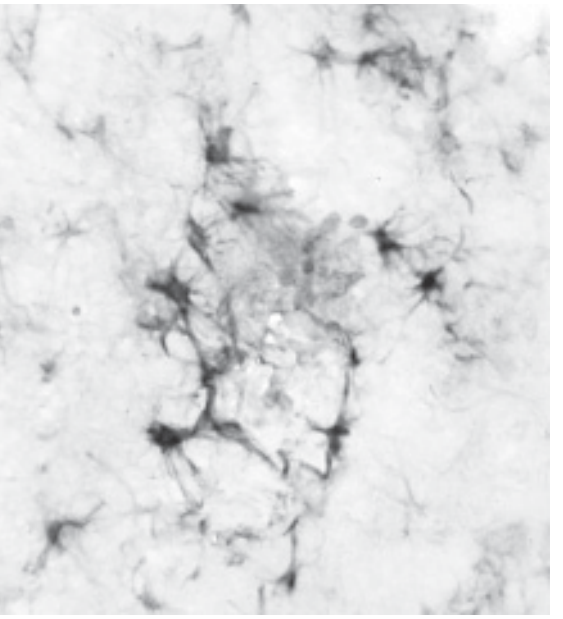

Fig. 1. This microphotograph demonstrates the close association of microglia, astroglia and an Aβ plaque in Alzheimer's disease. Microglia (grey) invade the center while astrocytes (black) wall off the plaque and are found in the periphery.

Aβ has been found in astrocytes of AD patients [55]. Like microglial cells, astrocytes are able to phagocytose Aβ, but they can do this spontaneously and without stimulation, as shown by Pihlaja and coworkers [62] who transplanted astrocytes into AβPP transgenic mice or applied them to human plaque containing brain sections.

Astroglial – microglial interactions

Astrocytes secrete immune modulators including CCL2, CXCL10, IL-6, IL-1β, IFN-γ and TGF-β [2, 18,22,50]. Several of these molecules are involved in pathways for astrocytic modulation of microglia.

For example, CCL2 (MCP-1) and CXCL10 (IP-10) are produced by astrocytes and may modulate activity as well as migration of microglial cells expressing the receptors CCR2 and CXCR3. This has been shown in demyelinating lesions of multiple sclerosis [78]. The importance of the ligand-receptor pair CCL2/CCR2 in AD has been demonstrated in an AβPP mouse model lacking CCR2. Together with a reduction in the number of microglia, Aβ clearance was reduced [19]. Fractalkine (CX3CL1) is produced by astrocytes and neurons. Its receptor is expressed exclusively by microglia [12]. Fractalkine secretion leads to inhibition of

neurotoxicity [53]. Deficiency of its receptor CX3CR1 resulted in increased neuronal damage in in vivo mouse models of neurotoxicity [12].

The pro-inflammatory cytokines IFN-γ and TNF-α, which can by produced by astroglia, stimulate pro-inflammatory microglial activation, while TGF-β and IL-1β modulate microglial activity towards an anti-inflammatory response and stimulate clearance of Aβ plaques [5,42,72].

Min et al. showed that when microglial cultures were treated with astrocyte conditioned media, iNOS expression and ROS production by the microglia were reduced [51]. They proposed that this effect was created by an increased expression of microglial antioxidant enzymes, such as heme oxygenase-1. The responsible factor in conditioned medium was identified as a small, heat labile active component possibly similar to neutrophil-producing soluble factor [51].

Therapeutic attempts to regulate pro- and anti-inflammatory states of glia

There is an increasing focus on the actions of microglia and astroglia in chronic neurodegenerative disorders. The question arises as to whether therapies can be found that influence the balance between pro- and anti-inflammatory activation states of these cells

It is well documented in epidemiological studies that non-aspirin NSAIDs lower the risk of AD (selected refs [30,41,47,77]). These studies exclusively involve classical NSAIDs and not the more recently introduced selective COX-2 inhibitors. The effect is dependent on NSAID use of at least 6 months and possibly two years or more. Clinical trials on AD patients have produced conflicting results. This may be because AD inflammatory treatment comes too late for significant beneficial effects to be observed. There may, however, be different reasons for the multiple clinical failures with selective COX-2 inhibitors. Brain is one of the few areas of the body that constitutively expresses high levels of COX-2 [75]. Such a high constitutive expression implies an important function in normal physiology. In addition to human clinical trial failures, it is noteworthy that several trials of selective COX-2 inhibitors in transgenic animal studies have also failed. In contrast, those with traditional NSAIDs have shown beneficial effects (reviewed by [49]). Other, COX-independent mechanisms, have been proposed to explain the epidemiological and animal model results, including peroxisome proliferator-activated receptor-gamma (PPARγ)-dependent microglia regulation [71] and direct effects on AβPP/Aβ processing and aggregation [20,29].

It has been shown in animal models of AD and MS, and in MS patients, that the ligand activated transcription factor PPARγ may be a target for affecting microglial regulation [27,28]. Activation of PPAR γ produces down regulation of microglial activity via IL-10, and even induction of microglial death [27,31,40,89]. Recent pilot trials with PPARγ agonists have suggested improved cognition and memory in AD patients [85].

TNF-α is a potent pro-inflammatory cytokine released by microglia. Its action can be blocked by binding to Etanercept (Enbrel), a biologic TNF-α inhibitor approved for treatment of rheumatoid arthritis. In a pilot trial of perispinal administration of Enbrel to AD patients, an improvement in MMSE, ADAS-Cog, and SIB was reported [79].

Endocannabinoid receptors may be another useful target to lower the harmful effects of inflammation. Treatment of cultured human microglia with ligands selecting for the CB2 receptor, but not the CB1 receptor, reduced neurotoxicity and inhibited secretion of IL-1β and TNF-α [34]. The CB1 receptor ligand WIN-55212-2 reduced the number of activated microglia and, at 1 mg/kg dose, potentiated the LPS-induced impairment of performance in the water maze test. Since CB1 receptors are not expressed by microglia and astrocytes, this effect was most likely due to interaction with neuronal cannabinoid receptors [44]. It was also demonstrated that Cannabidiol, a non-psychoactive cannabinoid found in hemp, reduced neuroinflammation in mice injected with Aβ. iNOS and IL-1β expression and release were inhibited [21].

Interleukin-1 is a pro-inflammatory cytokine, and increased levels aggravate injury. It has been shown that the endogenous IL-1 receptor antagonist (IL-1RA) can reduce brain injury caused by excitotoxic, ischemic and traumatic insults in rodent models. IL-1RA is being tested in a Phase II trial in stroke patients [43,74].

Interleukin-4 was shown to render microglia neuroprotective in hippocampal slices by down-regulation of TNF-α and up-regulation of IGF-1 [8,10].

For the best therapeutic outcome, it is important to start treatment before neurons develop tangles, degenerate and ultimately die. Accurate and sensitive diagnostic tools for early detection of AD are therefore highly desirable. In this respect, inflammatory biomarkers could prove to be useful tools. A recently published diagnostic test in blood was reported to identify AD with an accuracy of 89% [67]. When the same test was used to predict progression to AD in patients with

mild cognitive impairment (MCI), the overall accuracy was 81%. Eighteen signaling peptides were identified, belonging to two networks – one of them centered on immunological pathways (connected to TNF-α and M-CSF, monocyte-colony stimulating factor) and the other centered on the cell proliferation/differentiation pathway of epidermal growth factor (EGF) [67].

Metabolic syndrome is a chronic low-grade inflammation characterized by obesity, dyslipidemia, elevated blood pressure, insulin resistance, and high-normal levels of C-reactive protein. It has been shown to correlate with risk for a variety of age-related disorders such as diabetes, heart disease, and AD [68,81]. It has also been demonstrated that insulin resistance increases the risk of AD. In the insulin resistance syndrome, peripheral insulin levels are chronically elevated while brain insulin levels are reduced. This is associated with increased levels of Aβ and inflammatory agents. A correction of the abnormal insulin levels may reduce the risk of AD [13]. Simple life style choices have been shown to reduce the risk of the metabolic syndrome and, in turn, age-related disorders. In a study of over 8000 non-demented subjects it was shown that consumption of fruits, fish and omega-3 rich oils is associated with such a reduced risk of dementia [4].

SUMMARY

In summary, microglia are the main effecter cells for both innate and adaptive immune responses. They are in constant activity, continuously sampling their surrounding domain. When disturbed, they assume an activated state, usually responding to the problem by secreting an array of inflammatory mediators and toxins. Newer evidence indicates that activation may involve an anti-inflammatory state rather than an exclusively pro-inflammatory state. This is in analogy with Th1 and Th2 T-cell responses where anti-inflammatory rather than pro-inflammatory mediators are released. Microglia may have a greater capacity for phagocytosis in the anti-inflammatory mode. Aβ triggers a pro-inflammatory state which may impair the phagocytotic ability of microglia. Activation of microglia towards an anti-inflammatory state, as appears to be the case following Aβ vaccination in transgenic mice, might have therapeutic potential in AD and some other chronic neurodegenerative disorders. Astroglia as well as microglia may assume a pro- or anti- inflammatory state. In the pro-inflammatory state they secrete inflammatory cytokines and in the anti-inflammatory state a variety of neuroprotective growth factors. As with microglia, activation of astrocytes into an anti-inflammatory state may have therapeutic potential. Finding methods of tipping the scale for both microglia and astrocytes should be a productive area for future research.

ABBREVIATIONS

AD	Alzheimer's disease
ADAS-Cog AD	Assessment Scale-Cognitive Subscale
AFGF	Acidic Fibroblast Growth Factor
ALS	Amyotrophic Lateral Sclerosis
AβPP	Amyloid-β protein precursor
Aβ	Amyloid-β
BBB	Blood Brain Barrier
BDNF	Brain-derived Neurotrophic Factor
BFGF	Basic Fibroblast Growth Factor
C1q	C1q complement
CB1, CB2	Cannabinoid receptors 1 and 2
CCL	CC Chemokine Ligand
CCL2	Chemokine (C-C motif) Ligand 2
CCR2	Chemokine (C-C motif) Receptor 2
CD36	Thrombospondin Receptor
CD47	Integrin-Associated Signal Transducer
CIITA	Major Histocompatibility Complex Class II, Transactivator
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
COX-2	Cyclooxygenase-2
CX3CL1	Chemokine (C-X3-C motif) Ligand 1, Fractalkine
CXCL10	Chemokine (C-X-C motif) Ligand 10
CXCR3	Chemokine receptor CXCR3
EAE	Experimental Autoimmune Encephalomyelitis
ERK	Extracellular Regulated Protein-Serine Kinase
IFN-γ	Interferon-gamma
IGF-1	Insulin-Like Growth Factor 1
Ii	HLA Class II Invariant Chain
IL-10	Interleukin-10
IL-1RA	IL-1 receptor antagonist
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible Nitric Oxide Synthase
IP-10	Interferon-Inducible Protein 10
JNK	c-Jun-N-terminal Kinase
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MAC1	CD11b, Integrin, Complement Component Receptor-3
MAP kinase	Mitogen-Activated Protein Kinase
MCI	Mild Cognitive Impairment
MCP-1	Monocyte Chemoattractant Protein 1
M-CSF	Monocyte-Colony Stimulating Factor
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MMSE	Mini-Mental State Examination
MS	Multiple Sclerosis
MSR-1	Macrophage Scavenger Receptor 1

NGF	Nerve Growth Factor
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PGE-2	Prostaglandin E2
PPARγ	Peroxisome Proliferator-Activated Receptor-gamma
ROS	Reactive Oxygen Species
SIB	Severe Impairment Battery
STAT1	Signal Transducer and Activator of Transcription 1
TGF-β	Transforming growth factor-beta
Th1/Th2	Subsets of T-helper lymphocytes
THP-1	Human Monocytic Cell Line
TNF-α	Tumor Necrosis Factor-alpha
TREM2	Triggering Receptor Expressed on Myeloid Cells-2

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Section 3
Bacterial infection and Alzheimer’s disease

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Chlamydia Pneumoniae as an Etiologic Agent for Late-Onset Alzheimer's Disease

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Abstract. Sporadic, late-onset Alzheimer's disease (LOAD) is a progressive neurodegenerative disease that is now the most common and severe form of dementia in the elderly. That dementia is thought to be a direct result of neuronal damage and loss associated with accumulations of abnormal protein deposits in the brain. Great strides have been made in the past 20 years with regard to understanding the pathological entities that arise in the AD brain, both for familial AD (∼5% of all cases) and LOAD (∼95% of all cases). The neuropathology observed includes: neuritic senile plaques (NSPs), neurofibrillary tangles (NFTs), neuropil threads (NPs), and often deposits of cerebrovascular amyloid. Genetic, biochemical, and immunological analyses have provided a relatively detailed knowledge of these entities, but our understanding of the "trigger" events leading to the biological processes resulting in this pathology and neurodegeneration remains limited. For this reason, the etiology of AD, in particular LOAD, has remained elusive. However, a number of recent and ongoing studies have implicated infection in the etiology and pathogenesis of LOAD. This review focuses specifically on infection with *Chlamydophila* (*Chlamydia*) *pneumoniae* in LOAD and how this infection may function as a "trigger or initiator" in the pathogenesis of this disease.

Keywords: Alzheimer's disease, amyloid, *APOE*, *Chlamydia pneumoniae*, etiology, infection, LOAD, neuroinflammation

INTRODUCTION

The idea that idiopathic chronic diseases might be caused by, or exacerbated by, microbial infection has long been a focus of attention in modern western medical thought. For one example, early in the 1900's rheumatoid arthritis was considered to be an infectious disease, an explanation which faded in the 1930's but re-emerged over the course of the century [1–4]. Similarly, the development of multiple sclerosis is generally agreed to involve an infectious component [5], and congruent arguments have been proposed in relation to several other idiopathic chronic diseases. In all cases, infectious etiology and/or involvement in chronic disease initiation have been difficult to establish and thus have not been accepted by either the research or clinical communities. In place of viral, bacterial, or mycological agents, many alternative mechanisms have been examined to explain chronic disease genesis. For example, several large-scale studies have explored the possible genetic bases of rheumatoid arthritis and other chronic clinical entities, including late-onset Alzheimer's disease (LOAD). Not surprisingly, most such studies have indicated that disease development is not attributable to one or a few mutations or gene polymorphisms, as is the case in familial Alzheimer's disease (FAD). Rather, research indicates that disease genesis appears to be multifactorial, resulting from

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complex interactions between environmental factors and genetic background [6, 7].

Many attempts have been made to define associations between infectious agents and LOAD, but none have proved to be either etiologic or exacerbating for LOAD-related neuropathology. Viral pathogens targeted and so far dismissed include measles virus, lentiviruses, adenovirus, and several others [8, 9]. Importantly, studies from a group in the UK have identified herpes simplex virus type 1 (HSV-1) infection as a risk factor for development of AD in people expressing *APOE* ε4 [10, 11] (see also other material in this publication). In addition, various bacterial pathogens, including *Coxiella burnettii* and *Chlamydia trachomatis* have been investigated, but no relationship with AD neuropathogenesis was identified [12]. In earlier reports, we described an association between infection with the intracellular respiratory bacterial pathogen *Chlamydophila* (*Chlamydia*) *pneumoniae* and the genesis of LOAD [13, 14]. In this review, we summarize that work, as well as newer studies published by our group and others that implicate chlamydial involvement in the genesis of LOAD.

THE AMYLOID CASCADE HYPOTHESIS

The single idea that has predominated for almost three decades in studies of the neuropathology of AD is the "amyloid cascade" hypothesis. This hypothesis contends that the generation and deposition of amyloid-β (Aβ) are the critical events underlying neuronal degeneration [15]. It is applicable to FAD, since it is well established that this type of AD is caused by genetic mutations which result in increased amyloid formation and deposition. However, many studies have demonstrated that the etiology of LOAD does not originate from a small number of identical, similar, or other genetic defects, thus calling into question the generality of Aβ as the universal causative factor in AD. Importantly, the neuropathology underlying both FAD and LOAD is essentially identical, indicating that factors other than the genetic lesions underlying the former must exist to explain the neuropathology of the latter. LOAD typically presents in older age; indeed age is the primary risk factor for its development. Further, other risk factors have been proposed, including: atherosclerosis [16], Type 2 diabetes [17], neurotrauma [18], and infection [10, 13, 19]. Thus, a likely scenario for development of LOAD centers on a poorly understood interplay between genetic risk, as exemplified by possession of the *APOE* ε4 allele (see below), and environmental factor(s), including infection.

CHLAMYDOPHILA (CHLAMYDIA) PNEUMONIAE

C. pneumoniae is an obligate intracellular bacterial pathogen of the human respiratory tract that is responsible for community-acquired pneumonia [20]. This organism, like all chlamydial species, infects mucosal surfaces, in this case the lung/pulmonary and nasal mucosa [20–22]. Systemic dissemination of the bacterium from the respiratory tract has been documented [23], and available evidence indicates that the major vehicle for dissemination is the monocyte [24]. Epidemiologic studies show that *C. pneumoniae* is ubiquitous [25]. The organism undergoes an unusual biphasic developmental cycle during normal growth. In the first phase, the elementary body (EB), the infectious extracellular form of the organism, attaches to a target eukaryotic host cell; these are most often epithelial cells, but other cell types can be infected, including astrocytes, microglia, and neurons [14, 26, 27]. The organism then is endocytosed into a cytoplasmic inclusion within which it reorganizes into the metabolically active growth form of the organism, the reticulate body (RB). RB undergo several rounds of cell division, after which most reorganize back to the EB form. Newly-formed EB are released from the host cell *via* lysis or exocytosis to continue propagation of the infection [26].

Many studies have shown that under certain conditions and/or within specific host cell types the organism can and often does alter its biologic state to generate persistent, long-term infections. Chlamydiae undergoing such infections are morphologically aberrant and display an unusual transcriptional profile [28–30]. Importantly, reports indicate that the mechanisms of pathogenesis differ between active and persistent chlamydial infection, and it is in the persistent state that these organisms elicit chronic disease [31, 32]. *C. pneumoniae* has been associated with several chronic pulmonary diseases [33]. Infection with this organism has been associated with a wide array of non-respiratory diseases, including atherosclerosis, inflammatory arthritis, multiple sclerosis, and others [34–37]. While some of these associations remain controversial, the role of this organism in atherogenesis has gained significant credence during the last fifteen years [36, 38, 39].

C. PNEUMONIAE AND ALZHEIMER'S DISEASE

We first demonstrated DNA of *C. pneumoniae* in 90% of postmortem LOAD brain samples examined using specific polymerase chain reaction assays (PCR) [13, 14]; 5% of postmortem, age-matched, non-AD, control brain samples contained that DNA. Brain tissues from areas that typically display characteristic AD neuropathology were analyzed, including temporal cortex, hippocampus, parietal cortex, and pre-frontal cortex. Areas less often showing AD pathology, e.g., cerebellum, were included. In 17/19 LOAD brains, positive samples were obtained from at least one area with neuropathology, and in four cases, from the cerebellum. In the latter brains, severe neuropathology existed throughout, including the cerebella; in the two LOAD brains that were PCR-negative, mild pathology was observed [13]. Samples from PCR-positive brains were analyzed by immunohistochemistry and electron microscopy as well. LOAD samples contained *C. pneumoniae* antigens, particularly in the temporal cortex, hippocampus, parietal cortex, and pre-frontal cortex; perivascular macrophages, microglia, and astroglia all were immuno-positive for the organism. Electron microscopy of LOAD brain samples revealed chlamydial inclusions containing EB and RB. Immunoelectron microscopy demonstrated labeling of the organism with a monoclonal antibody to an outer membrane protein [13, 40]. Immunoelectron microscopy was negative in comparable PCR-negative control sections.

Frozen brain samples were analyzed by reverse transcriptase-PCR (RT-PCR) to determine if intact bacterial RNA was present. This analysis successfully targeted messenger RNA encoding the KDO transferase and a ~376 kDa protein specific to *C. pneumoniae*. Homogenates of representative PCR and RT-PCR positive samples were prepared and incubated with THP-1 cells in culture. Recovery of viable bacteria was successful from two different AD brains and negative from two control brains [13]. Thus, *C. pneumoniae* DNA and antigens were present in areas of neuropathology and the organism remained viable within frozen AD brain tissues. Additional analyses revealed that 11 PCR-positive samples had at least one allele for the *APOE* ε4 isoform (64%), consistent with that allele type being a risk factor for development of LOAD [41; see also below]. Importantly, a separate study in individuals with reactive arthritis showed that in patients who had *C. pneumoniae* DNA in their synovial tissues, 68% had at least one copy of the *APOE* ε4 allele. These observations implicated a relationship between the *APOEε4* allelic genotype and *C. pneumoniae*, and that together both factors confer increased risk for chronic disease genesis [13, 42].

Our initial studies and the implications of bacterial infection in the genesis of LOAD (see Fig. 1. Proof of Concept) led other groups to attempt identification of *C. pneumoniae* in tissue and other samples from patients with LOAD. Those studies provided mixed results, with some reports giving positive identification [43, 44], and others failing to find DNA or antigens from the organism in relevant samples [45–48]; many different techniques were used in these studies, with no other study using identical methodology to our own. In a previous review of the literature from other areas in which *C. pneumoniae* was implicated as a factor in disease genesis, discrepancies in analytical methods used among laboratories, and the variable data resulting from them, were pointed out [49]. It is clear that multiple reasons for these discrepancies are apparent.

We later extended our studies using new tissues from LOAD and non-LOAD control brains [14]. PCR analysis in multiple assays targeting two *C. pneumoniae* genes revealed that tissues from 20/25 LOAD brains, and from 3/27 non-LOAD control brains, were PCR-positive [14]. The organism was cultured from LOAD brains, and various chlamydial transcripts from additional LOAD brains, demonstrated the viability and metabolic activity of the organisms in those samples. Immunohistochemical analyses revealed that astrocytes, microglia, and ~20% of neurons were infected by *C. pneumoniae*. The finding of a large proportion of neurons PCR-positive for the organism in this later study was unique [14]. As in our initial study, infected cells were located in close proximity to both neuritic senile plaques and neurofibrillary tangle-containing neurons in the brain [13, 14]. These observations suggest a direct effect of *C. pneumoniae* on neuronal cell injury/death, as well as on the potential for the organism to act as a perpetrator/initiator of granulovacuolar degeneration in the LOAD brain.

Further analyses showed that intracellular and extracellular labeling for *C. pneumoniae* was present in the entorhinal cortex, the hippocampal formation, and the frontal cortex of all AD brains [50]. Serial sections from these areas exhibited both amyloid pathology and *Chlamydia* immunoreactivity in apposition to one another. Staining with

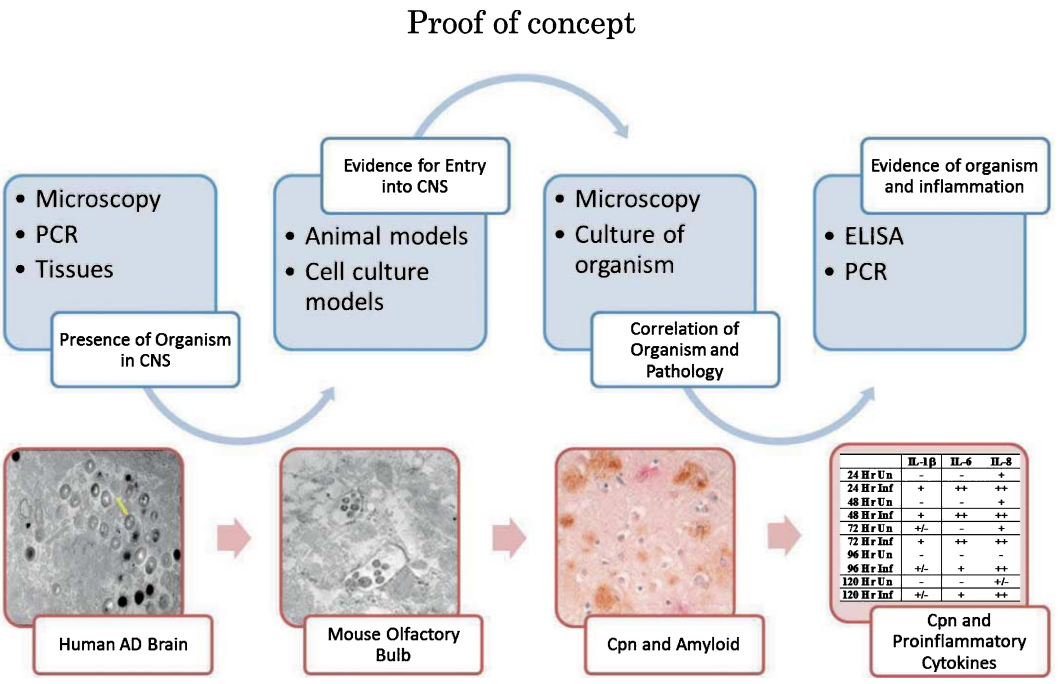

Fig. 1. Experimental protocols for proof of concept studies of *Chlamydia pneumoniae* in Alzheimer's disease. Using light and electron microscopy, molecular biology techniques (PCR and RT-PCR), and culturing of human brain tissues, *Chlamydia pneumoniae* was identified from Alzheimer brain tissues. Evaluation of the entry of *Chlamydia pneumoniae* into the CNS utilized animal models and *in vitro cell* culture models to demonstrate that olfaction and leukocyte infection resulted in CNS infection. Further analysis of both human AD brains and animal brains using a variety of microscopic and culture methods revealed the relationship between the presence of the organism and pathology characteristic of AD. Inflammation was determined to be one mechanistic process leading to damage in the CNS following infection using protein (ELISA) and molecular (mRNA microarrays) techniques. AD, Alzheimer's disease, PCR, polymerase chain reaction, RT-PCR, reverse transcriptase-polymerase chain reaction, CNS, central nervous system, ELISA, enzyme linked immunosorbent assay, mRNA, messenger ribonucleic acid.

Thioflavin S and anti-*C. pneumoniae* antibodies on the same sections revealed fibrillary amyloid and chlamydial immunoreactivity, respectively. Two extracellular patterns of chlamydial immunoreactivity were observed: a punctate pattern and an amorphous foci pattern. These likely represent extrusion of whole organism (punctate) or secreted chlamydial products, e.g., lipopolysaccharide (amorphous foci) [51, 52]. Amyloid has been shown to have anti-microbial properties, possibly allowing it to act as an anionic defensin [53–55]. These observations suggest that *C. pneumoniae* has a tropism for these brain regions, and that infection at these sites may be a precursor or trigger for development of damage. The olfactory structures, the entorhinal cortex, and the hippocampal formation are regions demonstrating the earliest damage in AD [56, 57]. The organism has been demonstrated in both human and animal olfactory bulbs [13, 58, 59]; in animals, the organism appeared to spread centrifugally from the olfactory bulbs into the brain proper [58–60]. Together, these and other observations given below support the idea that infection by this pathogen is an early event involved in the triggering of pathogenesis, and not a consequence of prior damage providing access of infection to the CNS.

C. pneumoniae is a respiratory pathogen, and this route for CNS infection is supported by studies in which the organism isolated from an AD brain was more closely related to respiratory than to atherosclerotic strains. As indicated, we prepared cultures of *C. pneumoniae* from LOAD brain tissues and determined molecular genetic and cell biological characteristics for two of them. Both isolates were genetically diverse (i.e., not clonal), as with most respiratory isolates [61]. Analyses for single nucleotide polymorphisms (SNPs) around the chromosome indicated several differences from standard respiratory isolates and strains, but we did not identify genetic attributes indicating a neurotropism. Recent full genome sequencing on one of the isolates confirmed this initial observation [62]. Cell biological

studies demonstrated standard inclusion morphology and chlamydial morphology of organisms of both isolates in human epithelial cells (HEp-2), astrocytes (U-87 MG), and microglial cells (CHME-5), as in our previously published studies [27].

C. PNEUMONIAE, *APOE*, AND LOAD

Several chronic diseases with which *C. pneumoniae* infection has been associated also are associated with possession of the ε4 allele type at the *APOE* locus on human chromosome 19 [5]. We provided evidence of a relationship between possession of that allele and infection with *C. pneumoniae* in LOAD. *In situ* hybridization analyses indicated that the number of *C. pneumoniae*-infected cells in affected brain regions of ε4-bearing LOAD patients was higher than that in congruent brain regions from LOAD patients lacking that allele [63]. Real time PCR analyses of brain tissues targeting DNA sequences from *C. pneumoniae* showed that the bacterial burden in samples lacking the ε4 allele varied widely, but that samples from ε4-bearing patients had significantly higher bacterial loads than did congruent samples from patients without the allele [63]. Other studies have elucidated the relationship between the *APOE* ε4 gene product, infection by *C. pneumoniae*, and disease genesis in several contexts. In all its forms, apoE is a secreted glycoprotein. Unlike apoE2 and apoE3, apoE4 enhances attachment of *C. pneumoniae* EB to host cells, including astrocytes and microglial cells, by about 3-fold over levels observed in the absence of that allelic product [64]. apoE4 adherent to the chlamydial EB retains its ability to attach to its normal receptor on the surface of host eukaryotic cells, i.e., the LDL receptor and other members of that receptor family [65;APH unpublished observations]. Thus, while we do not understand all details concerning how apoE4-enhanced host cell attachment is accomplished for *C. pneumoniae*, these observations provide a link between infection, apoE4, and clinical entities associated with both, including LOAD.

C. PNEUMONIAE, NEUROINFLAMMATION, AND LOAD

Chlamydia-induced disease is largely a result of immunopathogenesis. Chlamydial infection promotes secretion of proinflammatory cytokines [66]; strong inflammatory responses are engendered by chlamydial lipopolysaccharide (LPS), heat shock proteins, and outer membrane proteins. LPS alone could account for many aspects of LOAD pathology, as studies have shown that *E. coli* LPS, when injected at low dose into the brains of rats, results in inflammation characterized by increased cytokine production and microglial activation [67]. Comparable damage to that found in LOAD was observed in the rat temporal lobe (induction of the amyloid-β protein precursor) suggesting that products of infection produced by an organism, or by the host in response to it, stimulates inflammation leading to LOAD-related neurodegeneration.

In the LOAD brain, inflammation is thought to result from Aβ deposition, which has been advanced as the primary mechanism in LOAD pathogenesis [68]. Trials investigating the effects of non-steroidal anti-inflammatory drugs (NSAIDs) also implicate inflammation as a factor, since some have shown that these drugs can delay onset of LOAD [69]; however, they appear to be ineffective as a therapeutic for the disease. The resident cells in the brain responsible for inflammation are typically microglia and astroglia. Both are activated in the LOAD brain and often are identified in and around amyloid plaques [70]. Microglia and astroglia respond to insult by producing proinflammatory cytokines and reactive oxygen species (ROS). Identification of *C. pneumoniae* in the CNS in both cell types suggests that infection-initiated inflammation may be involved in LOAD neuropathology [13, 14]. We observed infected microglia, astroglia, perivascular macrophages, and neurons in areas of amyloid deposition. Activation of microglia and astroglia in response to infected, activated monocytes could promote increased production of a variety of cytokines and chemokines [71, 72]. Likewise, proinflammatory molecules were significantly higher in supernatant fluids of *C. pneumoniae*-infected murine microglial cells compared with controls [73]. Infected murine astrocytes showed higher levels of MCP-1 and IL-6 compared to controls. Neurons exposed to conditioned supernatant from infected murine microglial cells showed increased cell death compared with mock-infected supernatants; addition of neutralizing antibodies to IL-6 and TNFα to the conditioned supernatant reduced neuronal cell death by ∼50%.

Monocytes are altered with regard to expression of cytokines, apoptosis, and β−amyloid clearance in AD [74–78]. Our observations suggest that transcription of genes encoding inflammatory products changes significantly at 48 hr post-infection by *C. pneumoniae*, and that infected cells maintain

pro-inflammatory cytokine secretion over 5 days, including IL-1β, IL-6, and IL-8 [78]. High levels of IL-1β are correlated with neuroinflammation in the AD brain [79–82]. This cytokine activates nitric oxide synthase that has been implicated in hippocampal neuronal cell death [83, 84]. Further evidence has implicated IL-1β in promotion of the neuronal synthesis of the β-amyloid precursor protein [82]. These observations provide a rationale for triggering events by which Aβ would be a resultant factor in neuropathogenesis, not an initializing event.

Expression of four genes was significantly up-regulated after 48 hr infection in our studies; all encoded products are involved with host defense against bacteria [78]. *DEFB4* encodes a defensin protein with anti-microbial activity which links the innate and adaptive immune responses [85]. Interestingly, the β−amyloid 1–42 peptide in AD has been shown to act as an anionic defensin with antibacterial properties. Another important transcript up-regulated was that encoding inflammasomes. These are associated with toll-like receptors and mediate the response to both extracellular and intracellular pathogens [86]. Independent infection of monocytes with two strains of *C. pneumoniae* resulted in up-regulation of transcripts for two different inflammasome complexes, NLRC4 (IPAF) and AIM2. The former can be activated by type III secretion systems characteristic of *C. pneumoniae* and other gram negative bacteria [87]; this system acts to transfer effector proteins from the bacteria into the cytosol of the host cell, resulting in generation of ROS. ROS are thought to result in assemblage of another inflammasome complex, NLRP3 [88, 89] which also is activated by chlamydial infections [90, 91]. Furthermore, we observed up-regulation of the AIM2 inflammasome transcript, which would result in a complex activated by detection of double-stranded DNA in the cytosol [92, 93].

The transcript encoding MCP1/CCL2, a key chemokine for recruiting monocytes and macrophages, was increased up to 1000 fold following infection of monocytes with *C. pneumoniae;* [78, 94]; CCL2 was the most dramatically altered. This gene product is an important contributor to the neuroinflammatory process observed in AD and is increased in both CSF and plasma from individuals with mild cognitive impairment and AD [95, 96]. CCL2 may alter the blood brain barrier to allow increased monocyte migration into brain tissues, as well as affecting production and clearance of Aβ from the brain [95–97]. Thus, our studies demonstrating increased CCL2 production during *C. pneumoniae* infection in monocytes has implications for AD, since *C. pneumoniae* has been found in both the brain proper and in perivascular monocytes and macrophages [13, 14, 50, 98].

ANTIBIOTIC TREATMENT STUDIES

If infection by *C. pneumoniae* is involved in the genesis of LOAD, then antimicrobial treatment might be a therapeutic approach. A clinical trial has been reported that used a combination approach for treatment of LOAD [99]. The primary outcome was a change in Standardized AD Assessment Scale cognitive subscale (SADAScog) at 6 months. Secondary outcomes included changes in the SADAScog at 12 months and analysis of dysfunctional behavior, depression, and functional status. Results showed less decline in SADAScog score at 6 months in the antibiotic group compared to the placebo group; the SADAScog score at 12 months in the antibiotic and placebo groups was not significantly different. However, the antibiotic group showed significantly less dysfunctional behavior at 3 months, and at 12 months the antibiotic group showed reduced decline in mini-mental status scores. No correlations to change in criteria for *C. pneumoniae* infection were apparent as determined by analysis of serum antibody titers and PCR of blood samples. As with antibiotic trials to assess efficacy in obviating aspects of atherogenesis and cardiovascular disease, the outcome of the LOAD-related antibiotic trial indicated no meaningful efficacy in amelioration of relevant pathogenesis. These failures have been understood to mean that simple, straightforward antibiotic treatment of complex disease entities is not a viable strategy. It remains to be determined whether an antibiotic/anti-inflammatory regimen in at-risk patients or following early diagnosis could be effective.

Approaches other than antibiotic therapy may be helpful in treating AD. *C. pneumoniae* is known to persist in various contexts, and this persistent form is implicated in chronic diseases. One approach is to activate an immune response to eliminate intracellular infection. We used a synthetic peptide, acALY18 derived from an 18-mer sequence of the transient receptor channel protein 1(TRPC1) to treat *C. pneumoniae* infections of monocytes *in vitro* [100]; this peptide activates in part the NLRP3 inflammasome [101]. Using only a low dose of acALY18, only 12% of the cells remained infected at 24 h post-treatment, compared to 90% of cells left untreated

with the peptide [101]. At 48 h post-infection, 26 innate and adaptive immune transcripts were up-regulated in the infected/treated cells compared to infected/untreated cells. These transcripts occurred in four functional groups: (1) cytokines, chemokines, receptors, and signaling molecules; (2) host defense; (3) anti-bacterial response; and (4) modulators of the tissue response to inflammation. Future studies will address specific transcript up-regulation and protein expression leading to eradication of *C. pneumoniae* infection.

ANIMAL MODELS FOR *C. PNEUMONIAE* INFECTION

Previous models of AD have utilized transgenic mice that over-express mutants of presenilin and that for amyloid-β protein precursor [102]. Over-expression of amyloid results in development of amyloid plaques in the brain, paralleling the pathology observed in familial AD. However, these systems do not address the initiating events of LOAD, in which mutations of the amyloid-β protein precursor and presenilin are not present. We developed a non-transgenic animal model to address how infection might play a role in the pathogenesis of LOAD, independent of predisposing genetic factors [58]. This work utilized *C. pneumoniae* (AD brain isolate [13]) infection of naïve BALB/c mice to determine whether it would promote damage in the brain similar to that identified in sporadic LOAD. BALB/c mice are susceptible to a respiratory infection with *C. pneumoniae* (strain AR-39), and can maintain a persistent respiratory infection [103]. We tested the hypothesis that *C. pneumoniae* infection in BALB/c mice could initiate processes that result in the development of AD-like pathology in the brain [58].

Following intranasal infection, *C. pneumoniae* was identified in the olfactory epithelia (chlamydial antigens) and the olfactory bulbs (chlamydial antigens and typical morphology) by both light and electron microscopy [58]. Analysis of pathology in the brain revealed Aβ1–42 deposits that resembled amyloid plaques found in human AD. Activation of astrocytes and co-localization of some of these reactive astrocytes with the amyloid deposits suggested that a cellular inflammatory response was initiated. This response may be due to *C. pneumoniae* or directed against amyloid deposits or soluble amyloid induced by *C. pneumoniae* infection. These findings suggest that Aβ generation is a response to the infectious insult and lend support to the hypothesis that Aβ can act as a "bioflocculant" [104]. Induction of amyloid deposits in the brains of non-transgenic BALB/c mice supports the hypothesis that infection with *C. pneumoniae* can accelerate or induce AD-like pathology and may be a trigger in LOAD pathogenesis.

Further study was initiated to determine if antibiotic intervention following intranasal infection could treat or limit the pathology induced by infection in the CNS [105]. Following intranasal infection with *C. pneumoniae* (strain AR-39), mice were treated with moxifloxacin hydrochloride (Avelox) at days 7–21, 28–42, 56–70, or 84–98 post-infection; sacrifice was at 6 months post-infection with brains analyzed for *C. pneumoniae*, Aβ1–42 deposition (plaques), and astrocyte (GFAP) cellular reactivity. Immunohistochemistry analysis indicated that the organism was still present at 6 months post-infection in olfactory tissues and in the brain. At the earliest time of antibiotic treatment, the number of Aβ1–42 reactive amyloid plaques was equivalent to the level observed in uninfected mice. In the infected mice in which treatment was delayed until 56 days post-infection, the number of amyloid plaques was 8–9-fold higher than baseline; this was comparable to the number found in the brains of infected animals that received *no* antibiotics. These data suggest that early antibiotic intervention after infection is effective in limiting the amyloid plaques that arise as a result of infection, even though complete eradication may not be achieved. While more studies of early antibiotic treatment are underway, these results suggest that early intervention may be the most effective in limiting amyloid deposition.

RECENT ANIMAL MODEL STUDIES

Our recent studies used the AR-39 strain for infection, rather than *C. pneumoniae* isolates from the human brain. Brains were analyzed at 1–4 months post-infection by immunohistochemistry with *Chlamydia*-specific antibodies and antibodies specific for Aβ-amyloid 1–42 [106]. As in our report utilizing the brain isolate, no substantial amyloid deposits were observed at 1 month, and only limited AD-like pathology was identified at 2 months, post-infection. In contrast to the original study, at 4 months AD-like pathology was diminished; brains resembled those from mock-infected mice, suggesting that pathology had decreased 2–4 months post-infection. Interestingly, analyses

indicated that peak chlamydial burden preceded peak amyloid deposition by 1 month. These data suggest that *C. pneumoniae* infection serves as a primary stimulus for β-amyloid processing and subsequent deposition in brain tissues.

Precedents for infection in the exacerbation of AD-like pathology have been reported for other pathogens in other animal models [107, 108]. Once infection has been controlled, levels of soluble amyloid apparently decrease, resulting in fewer deposits at 3-4 months [109]. In mice infected with the brain isolate, our studies identified β-amyloid deposits as early as 2 months post-infection, with the greatest number of deposits identified at 3 months. AD-like pathology developed progressively as the number and size of amyloid deposits increased. Animal models that mimic sporadic LOAD have been hampered by the lack of understanding of primary factors that promote the early deposition of β-amyloid. However, models utilizing direct injection of microbial products have shown induction of transient amyloid production and deposition [110, 111]. One previous study did not identify substantial AD-like pathology in the brain following infection with a respiratory isolate/laboratory strain of *C. pneumoniae* [112]. The authors of that report noted that discrepancies could be due to the fact that the laboratory *C. pneumoniae* strain used may have different virulence properties than the human AD-brain isolate.

Our data indicate that isolates of *C. pneumoniae* differ in the ability to establish persistent infection and promote progressive neuropathology. A critical issue in development of sporadic LOAD is age, and by extension the age at which *C. pneumoniae* infection occurs. An earlier study from our group suggests that infection in older animals promotes establishment of a brain infection [59]. In other studies, aged C57BL/6 mice as compared to young counterparts had a greater propensity to develop chronic and/or progressive respiratory infections following intranasal infection with *C. pneumoniae* [113]. A heptavalent CTL epitope minigene vaccine conferred equal protection in the lungs of both aged and young mice. The vaccine partially protected against infection spread to the cardiovascular system of young animals but failed to provide protection in aged animals [113]. Our data suggest that vaccine strategies targeting the *C. pneumoniae*-specific CTL response are protective for respiratory infection in both young and old animals; however, the vaccine used was ineffective in preventing dissemination to the cardiosvascular system in aged mice or controlling replication of organism in these tissues [113]. On a related issue, we inoculated a small group of BALB/c mice with strain AR-39 either twice or three times at 30 day intervals, then sacrificed at day 90. Animals inoculated twice displayed 68, and those inoculated 3 times had 177, amyloid deposits (unpublished observations); mice receiving only a single intranasal inoculation showed an average of 17-18 deposits at 3 months post-infection. These preliminary observations suggest that multiple inocula of *C. pneumoniae* exacerbate pathology in the brain, which has implications for multiple exposures increasing risk for generating increased pathology in *C. pneumoniae*-infected AD patients.

CONCLUSIONS, FUTURE DIRECTIONS

It is clear from our observations and those of others summarized here that much remains to be elucidated regarding the fundamental biochemical, cellular, and molecular genetic underpinnings supporting the initiation and development of neuropathology of LOAD, and the possible involvement of *C. pneumoniae* in those processes (see Fig. 2. Cpn Infection Paradigm) Regarding the latter issue, host immune responses that limit or reduce *C. pneumoniae* replication and antigen burden may effectively decrease the organism as a primary stimulus for long-term production of β-amyloid. We suggest that the difference between progressive and non-progressive AD-like pathology is due to as yet uncharacterized differences between/among *C. pneumoniae* strains and the host genetic background in which those infecting strains operate. This implies that there are critically important different virulence factors including tissue tropism among *C. pneumoniae* isolates and strains. Thus, the ability of the organism to enter and persist in the CNS and potentiate a chronic inflammatory response will be critical to its role in the initiation and maintenance of AD pathogenesis. Future studies concerning infectious involvement in elicitation of AD pathology of course must include brains from mild cognitively impaired individuals, as well as additional non-AD cases, to assess when and where *C. pneumoniae* and/or other pathogens enter the brain. These investigations are important, since some control non-AD brains in our studies have shown chlamydial immunoreactivity associated with diffuse amyloid deposition, perhaps suggesting that CNS infection with *C. pneumoniae* is a prodromal event leading to neuronal damage prior to onset of AD.

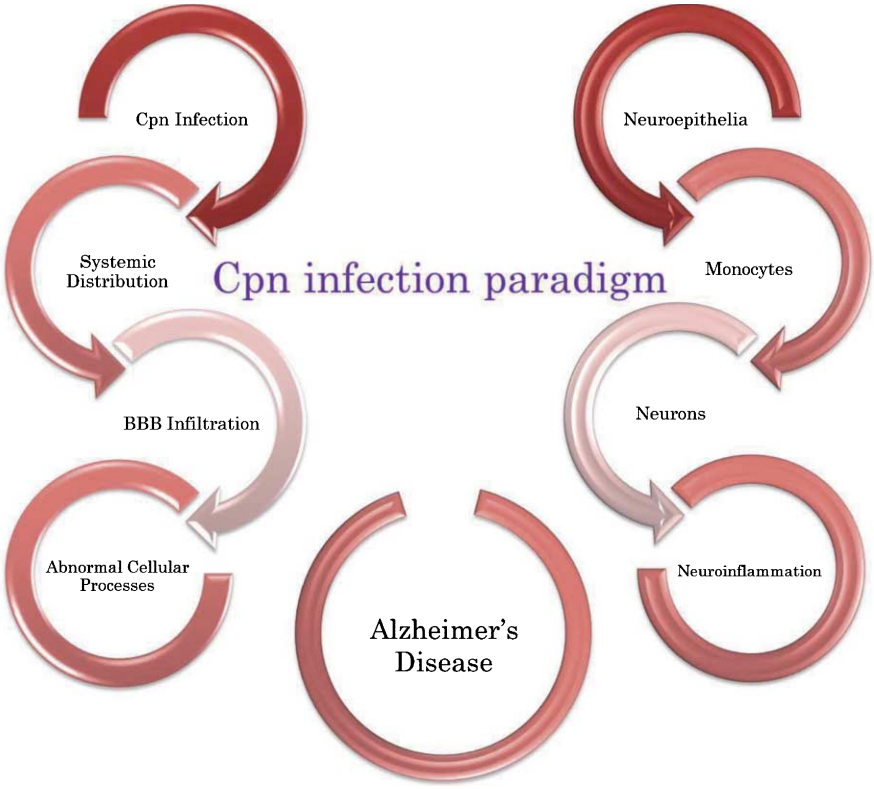

Fig. 2. *Chlamydia pneumoniae* infection paradigm for the etiology of late onset Alzheimer's disease. As a respiratory intracellular bacteria ubiquitous in nature, entry into the human respiratory tract begins following inhalation into the nose and lungs. Olfactory neuroepithelia in the upper nasal passages provides ready access of the infection into the olfactory pathways into the brain highlighting the selective vulnerability of this area. Organism inhalation into the lungs provides access to surveilling monocytes which engulf the organism and traffic back into the systemic circulation. Infection of both olfactory cells and monocytes results in eventual infiltration deeper into the brain where upon endothelial cells, astrocytes, microglia and neurons become infected. Presence of the infection in these cells and in the brain proper results in neuroinflammation and in abnormal cellular processes including the abnormal processing of the β amyloid precursor protein into Aβ amyloid.

We further suggest that, at this point in our understanding of AD, disease definitions usefully can be reconsidered in light of new observations from our group and many other sources. The most obvious neuropathologic aspects, and the disease phenotype, of LOAD are similar to, indeed apparently functionally identical to, those of early-onset disease. However, all studies to date consistently demonstrate that the former does not result from lesions in any of the three genes associated with the latter, and extensive research from many groups over the last thirty years has failed to identify any convincing mechanism by which the plaques and tangles are produced specifically in LOAD patients. Thus we have an etiologic conundrum – if the neuritic senile plaques and neurofibrillary tangles indeed are responsible in a direct manner for the neuronal death and consequent cognitive dysfunction that characterize both forms of the disease, it is not obvious how we can explain the genetic etiology of one but not the other form. One can argue that the plaques and tangles seen in LOAD are not caused directly by overtly pathologic mechanisms, such as those provided by lesions in *APP, PSEN1,* and/or *PSEN2*, but rather result from a gradual accumulation to neuropathologic levels as a function of normal biological/biochemical processes. However, those processes have not been identified to date despite an intense search for them, and in any case it has never been completely clear precisely how the neuritic senile plaques and neurofibrillary tangles underlie the neurotoxicity causing neuronal cell death in either early- or late-onset disease.

Current diagnostic criteria for LOAD add to the etiologic problem. At present, firm diagnosis results only from *post-mortem* examination of brain samples from candidate patients – disease definition is a function of the relative densities of neuritic senile

plaques and neurofibrillary tangles in relevant areas of the brain. The conundrum is made more profound by the fact that many individuals of advanced age show no signs or symptoms of dementia but possess plaque and tangle densities in excess of those accepted as characteristic of late-onset disease; conversely, other individuals tentatively diagnosed with LOAD on the basis of lack of evidence for Lewy-body involvement, vascular causation, or other problems underlying senile dementia can show low plaque and tangle densities [114]. In our view, these and other aspects of characterization and definition for each disease form require a rethinking of how causation is understood for each, and this is particularly imperative in the case of the increasingly prevalent late-onset clinical entity.

We therefore contend, in summary, that current evidence suggests that the early-onset form and the phenotypically similar, non-genetically-based late-onset form of dementia are in fact unrelated diseases. Clearly, early-onset disease has a genetic etiology, although the gene products of the affected genes may not elicit neuropathology exclusively by generation of the well-described plaques and tangles of the amyloid cascade hypothesis, but rather by other more subtle long-term developmental means. We suggest that the plaques and tangles may be an epiphenomenon in both early- and late-onset disease, and that they bear little or no responsibility for neuronal cell death that underlies the cognitive dysfunction. We suggest that, for both historical and biological reasons, early-onset disease can legitimately be designated Alzheimer's disease, but that the designation is not applicable or appropriate to the unrelated LOAD. The etiology of the latter not caused by vascular problems, Lewy body involvement, or other demonstrable origins remains to be elucidated, although it probably is a result of complex interactions between aspects of the genetic background of individuals at issue and any/several of a large number of possible environmental factors to which that individual has been exposed. Perhaps late-onset dementia should simply be referred to by that designation until a more detailed understanding of its causation is provided.

This proposed differentiation of early-onset dementia designated as Alzheimer's disease from late-onset disease is not, in our view, merely a superficial issue of formal categorization. Interestingly, late-onset Alzheimer's disease was classified originally as "senile dementia of the Alzheimer's type", based on endstage pathological findings of plaques and tangles without much understanding of mechanism or associated risk factors. Furthermore, as numerous insults to the brain can result in senile plaques, tangles, and/or other pathologies, to designate late-onset dementia as Alzheimer's disease is clearly inaccurate and thus untenable. Rather, we contend that the etiologies of early onset Alzheimer's disease and that of late-onset dementia are fundamentally different, and that progress in prevention and treatment of the latter, increasingly prevalent disease will be promoted by that distinction.

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Chronic Inflammation and Amyloidogenesis in Alzheimer's Disease – Role of Spirochetes[1]

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Abstract. Alzheimer's disease (AD) is associated with dementia, brain atrophy and the aggregation and accumulation of a cortical amyloid-β peptide (Aβ). Chronic bacterial infections are frequently associated with amyloid deposition. It had been known from a century that the spirochete *Treponema pallidum* can cause dementia in the atrophic form of general paresis where. It is noteworthy that the pathological hallmarks of this atrophic form are similar to those of AD. Recent observations showed that bacteria, including spirochetes contain amyloidogenic proteins and also that Aβ deposition and tau phosphorylation can be induced *in vitro* or *in vivo* following exposure to bacteria or LPS. Bacteria or their poorly degradable debris are powerful inflammatory cytokine inducers, activate complement, affect vascular permeability, generate nitric oxide and free radicals, induce apoptosis and are amyloidogenic. All these processes are involved in the pathogenesis of AD. Old and new observations, reviewed here, indicate that to consider the possibility that bacteria, including several types of spirochetes highly prevalent in the population at large or their persisting debris may initiate cascade of events leading to chronic inflammation and amyloid deposition in AD is important, as appropriate antibacterial and antiinflammatory therapy would be available to prevent dementia.

Keywords: Alzheimer's disease, β-amyloid, bacteria, Borrelia burgdorferi, chronic inflammation, dementia, general paresis, intestinal spirochetes, LPS, Lyme neuroborreliosis, neurospirochetosis, oral spirochetes, spirochetes, syphilis, Treponema pallidum

INTRODUCTION

Alzheimer discovered the disorder that bears his name a century ago, when he reported the case of a 51-year-old woman (Auguste D.) who suffered from presenile dementia with characteristic changes in the cerebral cortex [2, 3]. Alzheimer's disease (AD), the most common cause of dementia, is characterized by a slow, progressive decline of cortical functions, particularly cognition and memory. Terry and Davies (1980) [104] pointed out that the presenile form – with onset before age 65 – is identical to the most common form of senile dementia and suggested the term 'senile dementia of the Alzheimer type' (SDAT).

The pathological hallmarks of AD consist of a marked cortical atrophy, accumulation in the cerebral cortex of senile plaques (known also as argyrophylic or neuritic plaques), neurofibrillary tangles and neuropil threads. The occurrence of senile plaques was first reported by Blocq and Marinesco in 1892 [8] and the characteristic fibrillary changes of neuronal cells were first described and documented by Alzheimer (1907) [2, 3]. Recently, particularly from the use of Gallyas silver technique [24], the accumulation of neuropil threads or curly fibers has been recognized as a characteristic cortical lesion in AD.

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Fibrillary amyloid substance accumulates in senile plaques, but also in leptomeningeal and cortical vessel walls [26, 50]. The major subunit of the amyloid fibrils is the 4.2-kD amyloid-β peptide. The small self-aggregating peptide was designated as amyloid-β (Aβ) because of its partial beta-pleated sheet structure. Aβ is derived by proteolytic cleavage from a larger, transmembrane amyloid beta precursor protein (AβPP), which is expressed in a variety of tissues [41]. AβPP contains features characteristic of glycosylated cell-surface receptors and is revealed to be a proteoglycan core protein [87]. Neurofibrillary tangles contain paired helical filaments (PHFs) composed of the microtubule-associated protein tau. Tau is hyperphosphorylated in PHFs, which abolishes its ability to bind microtubules and promote microtubule assembly [27, 95]. The pathomechanism of Aβ and tangle formation still remains unclear. The role of chronic local inflammation in AD is well established, but the factors responsible for amyloid deposition and persisting inflammation are not known.

For about a century it has been known that chronic bacterial infection, caused by the spirochete *Treponema pallidum* in the atrophic form of general paresis in syphilis can cause dementia, brain atrophy and local amyloidosis. The possibility that microorganisms may play a role in the formation of senile plaques, was already discussed a century ago by Fischer, Alzheimer and their colleagues. Increasing recent evidences show that bacteria and their persisting remnants due to their biological activities may play a role in persisting inflammation and amyloid deposition in AD. The consideration that bacteria or their biologically active remnants may initiate the cascade of events leading to neurodegeneration brings together in a comprehensive way a large number of apparently diverse hypotheses, which have been proposed to play a role in the pathogenesis of AD. The old and new observations reviewed here indicate that to consider and support research on the role of pathogens in AD would be important, as appropriate therapy would be available. Joint antibiotic and anti-inflammatory therapies, if started early, may prevent or slow down the degenerative process.

PATHOGENESIS OF ALZHEIMER'S DISEASE

Although the first description of AD, which is the most frequent cause of dementia, dates back to a century ago, and despite of the enormous progress made in AD research, the elucidation of the cellular-molecular mechanisms involved in the degenerative process of AD is still unclear and the treatment unresolved [68].

A variety of scientific hypotheses were proposed to explain the pathogenesis of AD [5, 68, 88, 89, 92, 102].

Three genes are implicated in inherited forms of AD, with onset between ages 28–50 years. These are genes of AβPP located on chromosome 21, presenilin 1 (PS1) located on chromosome 14 and presenilin 2 (PS2) located on chromosome 1. The number of cases with these genetic mutations is low. There are less than 100 known individuals worldwide carrying the AβPP717 mutation [85, 103]. A fourth gene, apolipoprotein E (ApoE), is located on chromosome 19 and its E4 allele revealed to be a risk factor for late onset AD [86]. All these mutations appear to increase the production of Aβ. Finally, there is an association between AD and various polymorphisms in other genes, including a growing number of new genes implicated in immune defense mechanisms [53], which seem to have influence on the pathogenesis of AD.

The relation between Aβ and hyperphosphorylation of tau in AD is not yet fully elucidated. "Baptists" against "tauoists" claim the pathogenic role of Aβ versus tau. Extracellular, pre-amyloid Aβ protofibrils, versus intracellular Aβ accumulation for a direct role in AD pathology is discussed. The amyloid cascade hypothesis postulates that neurotoxicity of Aβ would cause the damage to neurons. Recent observations showed an interaction between Aβ and tau suggesting an important link between these major biological markers of AD [31], which is in agreement with previous observations that AβPP is an integral component of neurofibrillary tangles [77].

The role of ubiquitin; glycosylation end products; several neurotransmitters (e.g. the cholinergic hypothesis); hormones; neurotrophic factors, several metals, changes in calcium homeostasis and oxidative damage [70] to proteins, lipids and nucleic acids are other proposed alternative hypotheses. Several environmental factors; cardio-vascular risk factors such as cholesterolaemia, hypertension, cerebral hypoperfusion; mitochondrial abnormalities; disturbed signaling pathways (e.g. related to tau phosphorylation); are all factors which are implicated in the degenerative process in AD. Many other important factors not cited here, including cerebral cranio-cerebral trauma play an important role in the pathogenesis of AD.

CHRONIC INFLAMMATION IN ALZHEIMER'S DISEASE

Until recently, immune mechanisms in the pathogenesis of AD have been largely overlooked. Following the pioneer work of McGeer, Rogers and Griffin it is today generally accepted that cellular and molecular components of immune system reactions are associated with AD [28, 51, 55, 56]. Activated microglia (the brain's representatives of the phagocytic cells that are designed to clean up debris and foreign bacteria) surround senile plaques and extracellular neurofibrillary tangles. AD lesions are characterized by the presence of a series of inflammatory mediators, including cytokines, chemokines, proteases, adhesion molecules, free radicals, pentraxins, prostaglandins, anaphylatoxins, and activated complement proteins [52, 54].

It has been assumed that lymphocytic infiltration does not occur in AD. However, using specific immunohistochemical markers, both T-helper/inducer and T-cytotoxic/suppressor lymphocytes have been observed. Of particular importance is the association of the membrane attack complex (MAC, C5b-9) intended to lyse foreign cells, such as bacteria, with dystrophic neurites [55, 108]. The conclusion that inflammation exacerbates AD pathology is now supported by more than 20 epidemiological studies showing that individuals are spared AD if they have been taking anti-inflammatory drugs or have suffered from unrelated conditions for which such drugs are routinely used [56, 105]. This effect has been particularly evident in people using nonsteroidal anti-inflammatory drugs (NSAIDs). Three large epidemiological studies showed a reduction of risk of 55–80% for AD [96, 105, 113]. Further progression in this field of AD research is discussed in Chapter 2 of this book.

DEMENTIA, CORTICAL ATROPHY AND AMYLOID DEPOSITION CAUSED BY CHRONIC BACTERIAL INFECTION

Noguchi and Moor [69] were the first who demonstrated the persistence of *Treponema pallidum* spirochete in the brains of syphilitic patients suffering from general paresis. This important discovery established a direct pathogenic link between bacterial infection and dementia. Based on their observations it is now generally accepted that Treponema *pallidum* can cause chronic neuropsychiatric disorders including general paresis. In the long-standing or atrophic form of general paresis *Treponema pallidum* causes slowly progressive dementia, cortical atrophy, microgliosis and amyloid deposition. Intriguingly, the clinical and pathological hallmarks of the atrophic form of general paresis are similar to those occurring in AD (Fig. 1). Alzheimer himself referred to the similarity of the clinical picture in one of his AD patients with presenile dementia [3]. With respect to the histopathological changes, multiple authors have described *Treponema pallida* colonies confined to the cerebral cortex in patients with general paresis [37,

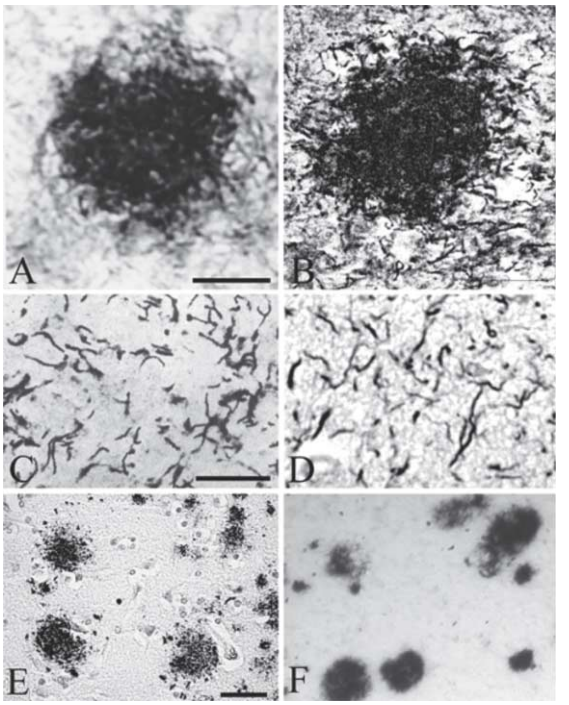

Fig. 1. The pathological hallmarks of Alzheimer's disease are similar to those occurring in the atrophic form of general paresis, a chronic bacterial infection caused by *Treponema pallidum*. A: Mass or colony of spirochetes visualized by the silver impregnation method of Dieterle for spirochetes in the cerebral cortex of a patient with general paresis. Reproduced by the kind permission of the publisher from R.R. Dieterle, Spirochetosis of the central nervous system in general paralysis, *Am. J. Psych.* **7** (1928), 37-67. B: Morphology of a senile plaque silver-stained with Bielschowsky technique for senile plaques. C and D show the similar morphology between silver impregnated Treponema pallidum spirochetes in the cerebral cortex of a patient with general paresis and cortical curly fibers or neuropil threads in a patient with sporadic Alzheimer's disease (Gallyas silver technique). E and F show similar distribution of beta amyloid in the cerebral cortex of a patient with the atrophic form of general paresis (E) and of a patient with Alzheimer's disease (F). Bars: A = 80 μm and is the same for B; C = 25 μm and is the same for D; E = 120 μm and is the same F.

38, 74, 75]. The morphology, distribution and histochemical properties of these colonies are identical to those of the senile plaques in AD. Senile plaques and spirochetal colonies both were described and called "miliary necroses" in the beginning of the last century [19, 98]. Neurofibrillary tangles have also been described in dementia paralytica [9, 78, 106] just as cortical and vascular amyloid deposition [107]. Recent characterization revealed that the aggregated amyloid substance corresponds to Aβ (Fig. 1, E) [67].

BACTERIA ARE POWERFUL STIMULATORS OF INFLAMMATION AND ARE AMYLOIDOGENIC

It is well known that several bacteria, on interaction with the mammalian immune system, induce chronic inflammation and amyloid deposition. Bacteria and their toxins are powerful inducers of inflammatory cytokines and activators of the classical complement pathway [22, 44]. It has been known from almost a century that chronic bacterial infections (*e.g.* rheumatoid arthritis, leprosy, tuberculosis, syphilis and osteomyelitis) are frequently associated with amyloid deposits in the infected tissues. It has also been known from almost a century that experimental amyloidosis can be induced by injecting living, attenuated or killed bacteria or bacterial components to experimental animals [79]. The bacterial inflammatory surface molecule lipopolysaccharide (LPS), a bacterial endotoxin is a powerful inflammatory and amyloidogenic factor of Gram-negative bacteria. LPS is used world wide in experimental *in vitro* and *in vivo* models of inflammation and amyloidosis. In bacteria (Prokaryotes), the cell wall consists of peptidoglycan, a complex polysaccharide composed of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid and a small group of amino acids, which comprise D-amino acids. Bacterial peptidoglycan is present only in bacteria, and is found in the wall of virtually all Eubacteria. It is absent in the evolutionary higher plant and animal cells (Eukaryotes). Poorly degradable "bacterial remnants" or alternatively, "dormant" fastidious bacteria may persist indefinitely in the affected organs [22]. LPS and bacterial cell wall peptidoglycan are highly resistant to degradation by mammalian enzymes and thus may provide a persisting inflammatory stimulus [71]. It has been shown that human intestinal bowel contains soluble bacterial cell wall components that are arthropathic in an animal model [97]. In these models it was the bacterial cell wall peptidoglycan component, which was found to be the arthritogenic factor [20].

SPIROCHETES

Spirochetes are Gram-negative free-living or host-associated helical bacteria possessing periplasmic fibrils, which are unique for these microorganisms. They are the causative agents *e.g.* of syphilis, Lyme disease, periodontitis, ulcerative gingivitis, and leptospirosis. *Treponema pallidum* is the pathogenic agent of syphilis. Many other Treponema species are found in the human mouth, uro-genital mucosa and gastrointestinal tract. Their pathogenic role is not yet fully established. *Borrelias* include *Borrelia burgdorferi*, the causative agent of Lyme disease; *Borrelia recurrentis* and *Borrelia vincentii* the causative agents of relapsing fever and Vincent's angina, respectively.

Treponema pallidum, which causes syphilis, is transmitted by sexual contact. *Treponema pallidum* has not yet been grown in synthetic media alone, although it has long been propagated in the testes of rabbits and cell monolayer systems [16]. *Borrelia burgdorferi*, which can be cultivated in a synthetic medium, is transmitted by tick bites to humans and causes Lyme disease [11]. The similarity of the clinical and pathological manifestations of syphilis and Lyme disease is striking [18]. *Borrelia burgdorferi* in analogy to *Treponema pallidum* can also persist in infected host tissues and play a role in chronic neuropsychiatric disorders. Dementia, including subacute presenile dementia, has been reported to occur not only in syphilis but also in Lyme disease [17].

ALZHEIMER'S DISEASE AND CHRONIC NEUROSPIROCHETOSIS

Nearly a century ago, Fischer has suggested that senile plaques may correspond to colonies of microorganisms [19]. Alzheimer cited Fischer's view in his discussion on the origin of senile plaques in AD [3].

Recent observations, using dark field microscopy analysis showed helically shaped microorganisms in the CSF, blood and cerebral cortex in 14 AD cases that were absent in 13 controls which were without any AD-type changes [59, 60]. Further taxonomic analyses have shown that these microorganisms possess axial filaments (endoflagellae) indicating that taxonomically they belong to the order Spirochaetales

[61, 62]. The amyloidogenic bacterial cell wall peptidoglycan was co-localized with Aβ in senile plaques in 17 AD cases analyzed and were absent in controls without any plaques or tangles [63, 64]. These results indicated that several types of spirochetes may be involved in AD including *Borrelia burgdorferi* and several types of oral and intestinal spirochetes [59–64].

It was MacDonald and Miranda [47] who first cultivated *Borrelia burgdorferi* spirochetes from the brain of two patients with concurrent AD and neuroborreliosis and proposed a possible link between AD and *Borrelia burgdorferi* [47, 48]. Miklossy [59] cultivated spirochetes in medium selective for *Borrelia burgdorferi* from the brains of 3 other AD patients where 16S rRNA gene sequence analysis identified the spirochetes as *Borrelia burgdorferi sensu stricto* (s. s.) [59, 65]. The post mortem serological analysis of blood and cerebrospinal fluid (CSF) and the detection of *Borrelia burgdorferi* specific antigens and DNA in the brains of these AD patients were further confirmations that these patients suffered from chronic Lyme neuroborreliosis. *Borrelia burgdorferi* specific antigens and DNA were co-localized with cortical Aβ deposits. The pathological findings were similar to those of the atrophic form of general paresis [37, 38, 75]. Consistent with these findings, the genospecies *Borrelia garinii* and *Borrelia burgdorferi s. s.* have been reported to be predominantly involved in neuroborreliosis [111]. Lyme disease is geographically confined and the incidence is low when compared to AD [12], which suggests that *Borrelia burgdorferi* is involved only in a low percentage of AD cases. The low number of cases investigated and the lack of a positive serology for *Borrelia burgdorferi* may explain why some previous investigators have failed to detect an involvement of *Borrelia burgdorferi* and AD [32, 49, 57]. In order to study the particular involvement of *Borrelia burgdorferi* in AD, it is important to analyze AD patients with a positive serology for *Borrelia burgdorferi*.

Antibodies to various "commensal" spirochetes, particularly spirochetes of the oral cavity are highly prevalent in the population at large [59]. Intestinal spirochetes were also cultivated from the blood of humans [21]. Riviere et al. [83] using species-specific PCR and monoclonal antibodies, detected oral Treponema spirochetes, which are known periodontal pathogens in 14/16 AD cases and in 4/18 controls. The invasive property of these oral Treponemes and periodontal pathogens was previously demonstrated [82].

Previous observations showed that in an analogous way to *Treponema pallidum*, *Borrelia burgdorferi* persists in the brain in chronic Lyme neuroborreliosis and following a long latent stage may lead to dementia, cortical atrophy and amyloid deposition [47, 48, 59, 60, 65, 66]. The presence of oral Treponemes in the brain in more than 90% of the AD cases analyzed [83] further suggests that these spirochetes may also persist in the brain and cause dementia and brain atrophy. Taken together, these observations strongly suggest that several types of spirochetes may sustain persisting inflammation and induce amyloid deposition in AD.

BACTERIA INDUCED Aβ DEPOSITION AND TAU PHOSPHORYLATION

Previous observations suggested that amyloidogenic protein may be an integral part of spirochetes and may play a role in amyloidogenesis in AD [59, 60, 66]. The more recent investigations made by Ohnishi et al. [72, 73] revealed that the outer surface protein (OspA) of *Borrelia burgdorferi* is amyloidogenic and forms amyloid fibrils *in vitro*, similar to human amyloid deposits. Recently, Aβ deposits were induced in rat primary neuronal and astrocytic cell cultures exposed to *Borrelia burgdorferi* spirochetes [66]. Using the reference strain B31 of *Borrelia burgdorferi* or strains ADB1 and ADB2, which were cultivated from the brain of AD patients had the same effect. Exposure of cultured mammalian neuronal and glial cells to these Borrelia spirochetes induced the defining pathological hallmarks of AD, including Aβ deposition, increased AβPP levels, and hyperphosphorylation of tau. Thioflavin S positive and Aβ-immunoreactive "plaques", as well as tangle- and granulovacuolar-like formations, were all observed in cell cultures exposed to spirochetes. Western blot analysis detected a 4kDa Aβ immunoreactive band in the infected cultures, which was more pronounced in microglia-enriched astrocytic cultures, suggesting that microglia may enhance Aβ formation. Using Synchrotron InfraRed MicroSpectroscopy (SIRMS) β-sheet protein structure was detected in the *in vitro*-induced Aβ deposits identical to that observed in senile plaques [66].

Increased AβPP levels were also detected in Borrelia-infected cultures, which may indicate the importance of host-derived AβPP in amyloidogenesis in AD. AβPP was shown to be a proteoglycan core protein [87, 112]. A role for proteoglycans

in the Major Histocompatibility Complex (MHC)-mediated infections is well established. The *in vitro* and *in vivo* synthesis of proteoglycans by host cells in response to bacterial infections, including spirochetal infections, has been repeatedly reported [101]. Proteoglycans are present in early stages of all type of amyloid formations [94] but their exact role in amyloidogenesis has yet to be determined. Increased tau phosphorylation detected in cell cultures exposed to Borrelia spirochetes represented further experimental evidence, which together with Aβ deposition and increased AβPP levels supported the role of bacteria mediating amyloidogenesis in AD [66]. These observations suggest that spirochetes may play a role in amyloid formation and participate in the development of the defining morphological changes of AD.

Infusion of LPS for 37 days into the 4th ventricle of rats can reproduce many of the inflammatory, neurochemical, neuropathological and behavioral changes seen in AD [33]. Aβ accumulation and increased AβPP mRNA in the basal forebrain and hippocampus was observed in response to LPS infusion [33, 34]. Infusion of LPS induced Aβ deposits and activation of microglia alleviated by ibuprofen [81]. LPS induced acceleration of amyloid deposition in LPS-treated APPV717F transgenic mice was also reported [80]. LPS-induced-neuroinflammation increases intracellular accumulation of AβPP and Aβ in APPswe transgenic mice [91].

In addition to increased AβPP levels hyperphosphorylation of tau was also observed following exposure of primary astrocytes to LPS [66]. It was shown that LPS stimulates the secretion of the AβPP via a protein kinase C-mediated pathway [93]. These observations indicate that not only living bacteria, but natural or synthetic bacterial components alone may also have important biological activities in mammals. Aβ secretion by a microglial cell line was induced by Aβ-25-35 and by LPS [7] suggesting an important role of microglia in Aβ aggregation and accumulation in AD. Microglial production of Aβ may be increased by proinflammatory stimuli or by Aβ itself.

Increasing number of recent observations show that several bacteria contain amyloidogenic proteins [6, 13, 15, 25, 40]. Analysis of the periplasmic outer membrane lipoprotein – OsmB – of Escherichia coli showed a similarity in amino acid sequences to Aß peptide [40]. Recent biochemical, biophysical, and imaging analyses revealed that fibers produced by Escherichia coli, termed "curly" were composed of amyloid [15].

Reports of associations between infection and AD are not confined to spirochetes. The presence of Herpes virus type 1 (HSV-1) in the AD brain has been reported [35, 36, 39]. *Chlamydia pneumoniae* was also found to be associated with AD [4] and mice exposed to Chlamydia developed AD-like amyloid plaques [45]. Amyloid deposits resembling plaques found in Alzheimer's disease (AD) brains were formed in the brains of non-transgenic BALB/c mice following intranasal infection with Chlamydia pneumoniae [45], indicating that several bacteria may induce Aβ deposits.

However, it is noteworthy that the clinical and pathological hallmarks of Alzheimer's disease (AD) as illustrated by historic literature are similar to those of the atrophic form of general paresis caused by *Treponema pallidum* spirochetes (Fig. 1) [37, 38, 46, 74, 75]. We should also consider that co-infection of spirochetes with other bacteria, including *Chlamydia pneumoniae* and Herpes viruses and also Candida albicans is frequent. The accumulation and persistence of bacteria and/or their degradation products in host tissues through their toxic component and amyloidogenic proteins may trigger a cascade of events leading to chronic inflammation and amyloid deposition.

BIOLOGICAL ACTIVITIES OF BACTERIA INDUCING AN ALZHEIMER'S TYPE HOST REACTION – A UNIFYING HYPOTHESIS?

The view that bacteria may play a role in the pathogenesis of AD would be in harmony with the majority of hypotheses proposed to play a role in the pathogenesis of AD. It does not contradict that genetic defects occur in AD. There is accumulating evidence that host responses and susceptibility to bacterial infections are genetically controlled [1, 90]. The genetic mutations occurring in AD (AβPP, Presenilin1 and 2) are all related to the processing of the AβPP. AβPP, a proteoglycan core protein, plays a role in cell defense mechanisms. As the production of proteoglycans aims to decrease infection, genetic defects of AβPP, PS-I and PS-II may be associated with an increased susceptibility to infection.

Mammals are constantly exposed to bacteria. Biologically active bacterial cell components are highly resistant to degradation by mammalian enzymes and thus may provide a persisting inflammatory and amyloidogenic stimuli [22, 23]. The innate immune

system, particularly the host complement system, plays an important role in the elimination of invading pathogens. Bacteria, similarly to Aβ, activate both the classic and the alternative complement pathways [10, 84], which through the common membrane attack pathway, results in bacteriolysis. Specific acquisition of different host plasma proteins, *e.g.* coating their surfaces with host complement regulators, such as factor H, allows pathogens evading from host complement attack and phagocytosis, and to persist in affected host tissues. Characteristic features of *Borrelia burgdorferi sensu lato (s.l.)* group are their ability to invade tissues and to escape complement lysis despite elevated levels of Borrelia-specific antibodies in serum and other body fluids. *Borrelia burgdorferi* prevents complement attack by binding the complement inhibitors factor H (FH) and factor-H-like protein-1 (FHL-1), the two major regulators of the alternative complement pathway, to their surfaces. Surface-attached FH and FHL-1/reconectin maintains its complement regulatory activity and promote factor I-mediated C3b cleavage to iC3b preventing bacteriolysis by the alternate complement pathway (Fig. 2). Complement resistant strains of *Borrelia burgdorferi* possess five complement regulatory acquiring surface proteins (CRASPS) that specifically bind FH and FHL-1 [43, 43]. Bacteria evading from complement lysis will survive and proliferate in affected tissues, with consequent accumulation of persistent biologically active bacterial debris and through a vicious circle may sustain inflammation and amyloid deposition. Accordingly, both, the classic and alternative complement pathways are activated in AD and critical components of both pathways, including factor H are associated with cortical lesions and activated microglia [99, 100].

In addition, bacteria are powerful inflammatory cytokine stimulators, they affect vascular permeability, they generate nitric oxide, and they induce proteoglycan synthesis and apoptosis [22, 23, 33, 34]. Exploding number of observations related to the mechanisms involved in *Treponema pallidum* and *Borrelia burgdorferi* infections indicate that exposure of host to spirochetes or to their toxic products, through a complex interaction with the host immune

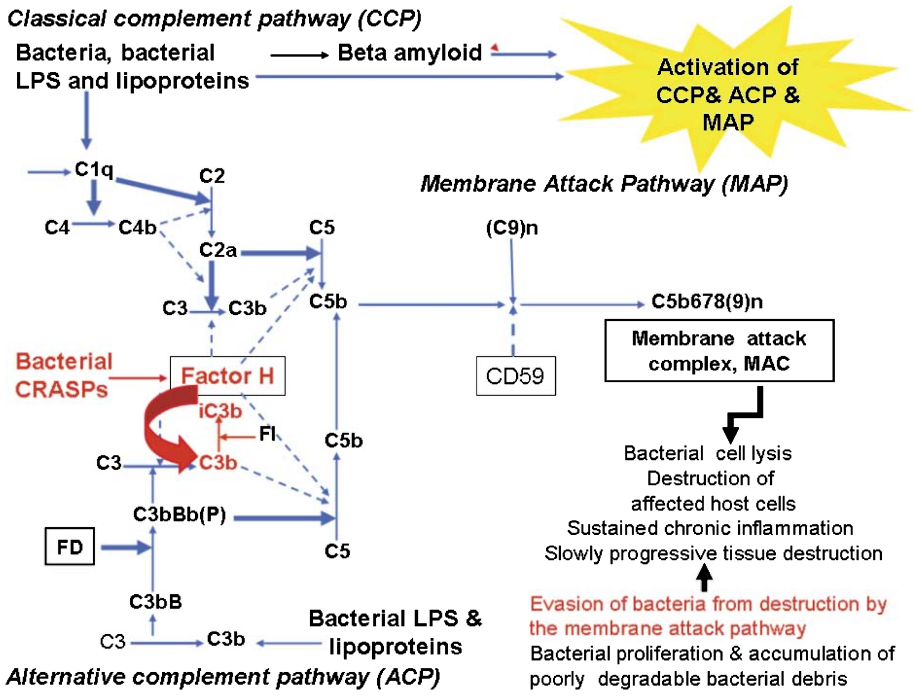

Fig. 2. Bacteria and beta amyloid are both able to activate the classic and the alternate complement pathways (CCP, ACP) through the common membrane attack pathway (MAP) resulting of bacteria and affected host cell lysis by the membrane attack complex (MAC or C5b-9). One way of evasion of Bacteria from complement lysis is their ability to bind the complement regulatory protein, factor H of the alternate pathway. Complement resistant *Borrelia burgdorferi* strains possess complement regulatory acquiring surface proteins (CRASPs), which specifically bind factor H, resulting in inactivation of C3b (iC3b) and in evasion of spirochetes from bacteriolysis by C5b-9 (MAC). Continuous arrows = activation, interrupted arrows = inhibition.

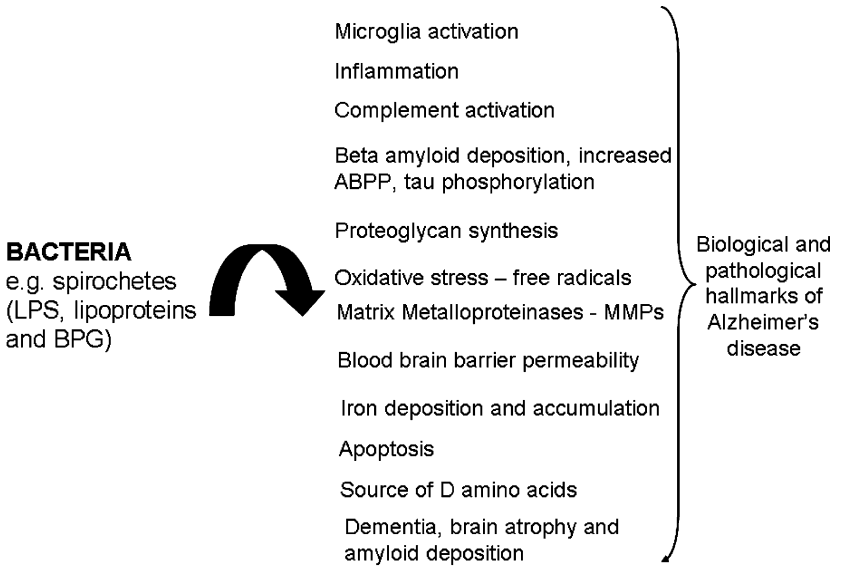

Fig. 3. Bacteria induced Alzheimer's type host reactions.

responses may induce persistent chronic inflammation, leading to slowly progressive tissue destruction.

One of the characteristic lesions of paretic dementia is the accumulation of iron in infected brain tissue [58]. Iron is essential for bacterial growth, and is recognized to play a vital role in infection. Iron has been shown to increase the formation of reactive oxygen intermediates leading to lipid peroxidation and subsequent oxidative damage to proteins and nucleic acids. Iron also affects the antigen-specific cellular responses by affecting T cell generation, T cell functions and proinflammatory cytokine production by macrophages [29, 30, 109, 110]. *Borrelia burgdorferi* contains a transferrin-binding protein suggesting that host transferrin (Tf) may be the source of iron for this spirochete [14]. *Borrelia burgdorferi* also induces Matrix Metalloproteinases (MMPs) [76]. All of these processes are implicated in the pathogenesis of AD (Fig. 3).

Bacteria or their biologically active toxic components may both induce Aβ accumulation and tau phosphorylation.

CONCLUSION

The pathological hallmarks of AD consist of Aβ plaques and neurofibrillary tangles in affected brain areas. The processes, which drive these host reactions are unknown. It has been known from one hundred years that chronic bacterial infection may lead to amyloid deposition not only in naturally occurring infections (e.g. syphilis, tuberculosis, leprosy, osteomyelitis) but also following injection of bacteria to experimental animals. In 1913, Noguchi and Moor showed the persistence of spirochetes in the brain of syphilitic patients suffering from dementia paralytica. This observation established a direct link between dementia and chronic bacterial infection. Today it is generally accepted that *Treponema pallidum* is responsible for dementia, brain atrophy and amyloid deposition in the atrophic form of general paresis in syphilis and also that this spirochete can cause several other neurodegenerative disorders.

Increasing number of recent evidence show that several spirochetes, including *Borrelia burgdorferi* and oral Treponema may be involved in the pathogenesis of AD. They may persist in the brain and following a long latent stage in an analogous way to *Treponema pallidum* may cause dementia, cortical atrophy and amyloid deposition. Historical and recent data available indicate that to consider the view that bacteria may trigger a cascade of events leading to chronic inflammation and amyloid deposition is important as one may prevent or stop the disease with an appropriate antibiotic and anti-inflammatory therapy.

Bacteria are powerful stimulators of inflammation; they are amyloidogenic and posses biological activities, which can induce the cascade of events leading to the pathological and biological hallmarks of AD. The purpose of this review was to show that the accu-

mulated knowledge, views and hypotheses are not lying so far from each other. Each of them has its own importance and they form together a comprehensive entity when observed in the light of a persisting chronic inflammation sustained by bacteria infection or their persisting remnants.

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Borrelia Burgdorferi Persists in the Brain in Chronic Lyme Neuroborreliosis and may be Associated with Alzheimer Disease

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Abstract. The cause, or causes, of the vast majority of Alzheimer's disease cases are unknown. A number of contributing factors have been postulated, including infection. It has long been known that the spirochete Treponema pallidum, which is the infective agent for syphilis, can in its late stages cause dementia, chronic inflammation, cortical atrophy and amyloid deposition. Spirochetes of unidentified types and strains have previously been observed in the blood, CSF and brain of 14 AD patients tested and absent in 13 controls. In three of these AD cases spirochetes were grown in a medium selective for Borrelia burgdorferi. In the present study, the phylogenetic analysis of these spirochetes was made. Positive identification of the agent as Borrelia burgdorferi sensu stricto was based on genetic and molecular analyses. Borrelia antigens and genes were co-localized with beta-amyloid deposits in these AD cases. The data indicate that Borrelia burgdorferi may persist in the brain and be associated with amyloid plaques in AD. They suggest that these spirochetes, perhaps in an analogous fashion to Treponema pallidum, may contribute to dementia, cortical atrophy and amyloid deposition. Further *in vitro* and *in vivo* studies may bring more insight into the potential role of spirochetes in AD.

Keywords: Alzheimer's disease, amyloid, bacteria, *Borrelia burgdorferi*, chronic inflammation, Lyme disease, spirochetes, syphilis

INTRODUCTION

The patho-mechanism of amyloid formation in Alzheimer's disease (AD) remains unclear. A combination of genetic predisposition and environmental factors may contribute to changes in amyloid precursor protein (AβPP) expression and amyloid beta peptide (Aβ) formation. Inflammatory processes play a crucial role in the development of AD [27]. Bacteria or bacterial components (e.g. bacterial lipopolysaccharide – LPS) are powerful activators of inflammatory processes and are known to stimulate amyloidogenesis.

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Increasing recent data support the possibility that infectious agents may play a role in AD [2, 7, 17, 18, 20, 23, 25, 29–33, 44]. Chronic bacterial infections, caused by spirochetes *such as Treponema pallidum* are known to be associated with chronic neuropsychiatric disorders including dementia. Spirochetes are Gram negative free-living or host-associated helical bacteria, possessing periplasmic fibrils which are unique for these microorganisms. They are widespread in aquatic environments and are the causative agents of *such* important human diseases *as* syphilis, Lyme disease, periodontitis, ulcerative gingivitis, and leptospirosis. *Treponema pallidum*, the causative agent of syphilis, is a tightly spiralled spirochete (about 0.1 μm $\times$ 20 μm) transmitted by sexual contact. *Treponema pallidum* has not yet been grown in synthetic media alone, although it has long been propagated in the testes of rabbits and recently in cell monolayer systems [12], as reviewed previously [9].

Borrelia burgdorferi, which can be cultivated in a synthetic media, is a larger (0.1–0.3 μm $\times$ 30 μm) spirochete, which is transmitted by tick bites to humans and causes Lyme disease. They both belong to the family Spirochaetaceae.

In the tertiary form of syphilis known as general paresis, *Treponema pallidum* persists in the brain and can cause cortical atrophy, microgliosis, and amyloid deposition [38, 39, 46]. There is a similarity in the clinical and pathological manifestations of syphilis and Lyme disease which are both caused by spirochetes [11]. *Borrelia burgdorferi* may also persist in infected host tissue and play a role in chronic neuropsychiatric disorders. Dementia, including subacute presenile dementia, has been reported to occur not only in syphilis but also in Lyme disease [10].

Intriguingly, the clinical and pathological hallmarks of AD are also present in the atrophic form of general paresis [19, 39]. Alzheimer himself referred to the similarity of the clinical manifestations of AD and general paresis, when he described one of his famous cases in 1911 [1]. In 1907, Fischer suggested that senile plaques may correspond to colonies of microorganisms [13].

Previously we reported helically shaped microorganisms in the cerebrospinal fluid (CSF), blood and cerebral cortex of 14 AD cases that were absent in 13 control cases [29]. An ultrastructural study indicated that the microorganisms taxonomically belong to the order Spirochaetales [30]. In three of these 14 AD cases spirochetes were grown in a medium selective for Borrelia burgdorferi. We suggested that several types of spirochetes may be involved in AD, including Borrelia burgdorferi. Subsequently Riviere et al., using species-specific PCR and monoclonal antibodies, detected oral Treponema in 14/16 AD cases and 4/18 non-AD controls [44].

We analyzed the sequence of the16S rRNA gene of the spirochetes grown in medium selective for *Borrelia burgdorferi* and carried out morphological characterization by transmission electron microscopy. Since diagnostic and serological tests are available for Borrelia burgdorferi, we correlated this with post mortem serological analysis of blood and cerebrospinal fluid (CSF) and were able to detect Borrelia burgdorferi antigens and genes in brain samples from the same cases where the spirochetes were cultivated. The molecular analysis of spirochetes cultivated from the blood of a clinically asymptomatic forester who showed positive serology for Lyme disease was also performed. As a control, a previously characterized reference, B 31 strain of Borrelia burgdorferi was utilized for comparative genomic characterization.

MATERIAL AND METHODS

Patients, clinical data

Previously we reported helically shaped microorganisms in the cerebrospinal fluid CSF, blood and cerebral cortex in 14 AD cases that were absent in 13 control cases [29]. An ultrastructural study showed that these microorganisms belong to the order Spirochaetales [30]. In 3 of these 14 AD cases spirochetes were cultivated from the brain in a synthetic BSK II medium using serial subcultures. According to clinical records, these patients suffered from AD type dementia. The age of the patients was 74, 78, and 86 years, and the cause of the death was rupture of an aortic aneurysm, cardiac failure, and bronchopneumonia, respectively. In case AD2 the clinical records mentioned traumatic brain injury 8 years before death. These three AD patients were living in the western (French-speaking) geographic area of Switzerland where Lyme borreliosis is endemic and is responsible for much systemic morbidity [36].

Spirochetes were also cultivated from the blood of a forester, a healthy blood donor (HF), whose serological tests were positive for *Borrelia burgdorferi*. Blood and serum samples as well as the cultivated spirochetes from this *latter* patient were also available for analysis.

A semiquantitative analysis of the density of senile plaques and neurofibrillary tangles in the AD cases

was performed in the hippocampus and entorhinal cortex, as well as in the frontal and parietal associative areas, as previously described in detail [34]. The 3 AD cases with dementia fulfilled criteria for *a* definite diagnosis of AD. The neuropathological assessment of the severity of cortical involvement was also made following Braak and Braak criteria [4]. For the neuropathological diagnosis of AD, the criteria recommended by Khachaturian [22], CERAD [35] and the National Institute on Aging (NIA) – Reagan Institute Working group were fulfilled [37].

Since the epsilon-4 allele of apolipoprotein E (Apo-E) is an important risk factor for AD, genotyping of Apo-E was performed in the three AD cases analyzed in this study. DNA was extracted from frontal cortical samples (about 25 mg) using Quiagen DNA extraction Kit (Quiagen, 29304) following the instruction of the manufacturer. Amplification of the human Apo-E gene and restriction enzyme isotyping with Hha-I was performed as described by Hixson and Vernier [16]. Following cleavage with Hha-I, 1 μl samples were run on polyacrylamide gel (Phastgel Pharmacia, 8–25%, 17-0542-01) in Phastsystem electrophoresis (Pharmacia Biotech). DNA bands were revealed by silver impregnation using an automated program of Phastsytem (Pharmacia Biotech). The solution for silver impregnation and the developer were prepared following the instructions of the manufacturer. Cases with known Apo-E genotypes 3/3, 3/4, and 2/4 were analyzed in parallel and used as internal controls.

Molecular characterization of the cultivated microorganisms

The comparative sequence analysis of the 16S rRNA gene sequences in the spirochetes isolated from the two AD brains and the healthy forester was carried out. Comparative sequence analysis of the 16S rRNA in the spirochetes isolated from the two AD brains and the healthy forester was carried out. Comparative analysis of 16S rRNA gene sequences is presently considered to be the gold standard for bacterial identification. 16S rRNA is a highly conserved molecule that is present in all prokaryotic organisms. It exhibits functional constancy and its sequence has evolved slowly, that allow most phylogenetic relationships to be measured [48], Other conserved genes do not necessarily meet these criteria.

DNA was isolated from cultured spirochetal cells and PCR amplified using the universally conserved primers previously described [41]. As a negative control, buffer containing no amplifiable DNA was utilized. Cycling conditions were followed as previously described [41]. A spirochetal selective reverse primer C90 (5′-GTT ACG ACT TCA CCC TCC T-3′) was used with a universal forward primer C75 (5′ GAG AGT TTG CTG GCT CAG-3′). Three μl of the crude DNA and 1 μM of primers were added to the reaction mixture, which had a final volume of 82 μl. Ampliwax PCR Gem 100's was used in a hot-start protocol as suggested by the manufacturer. The following conditions were used for the amplification using primers C70 and B37: denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, and elongation at 72°C for 90 sec with 5 additional sec added for each cycle. A total of 30 cycles was performed followed by a final elongation step at 72°C for 15 min. Conditions for amplification using primers C90 and C75 were identical, except that the annealing temperature was 60°C. After removal of Ampliwax, 0.6 volumes of 20% PEG 8000 (Sigma) in 2.5 M NaCl were added, and the mixture was incubated at 37°C for 10 minutes to precipitate the DNA. The sample was centrifuged for 15 minutes at 15 000 g and the pellet washed with 80% ethanol. The pellet was then dissolved in 35 ml of sterile water.

Sequencing and 16S rRNA data analysis followed those described by Fox et al. [14]. The DNA sample from PCR after purification was directly sequenced using cycle-sequencing kits (TAQuence Cycle Sequencing kit, USB, Cleveland, OH) or an fmol DNA Sequencing kit (Promega Corp.). Primers were end-labeled with 33P-ATP (NEN-Dupont) using the manufacturer's protocol. Twenty-five to 80 ng of purified DNA from the PCR *amplification* was used for each sequencing reaction. Reaction products were run electrophoretically on 8% polyacrilamide-urea gels and were subsequently detected by exposure of the dried gels to X-ray film for 24 to 48 h.

16S rRNA sequence analysis

Programs for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and phylogenetic tree construction were written in Microsoft QuickBASIC for use on IBM PC-AT and compatible computers. Our sequence database contains approximately 1000 sequences as determined in our laboratory" [43]. The sequences of most of the cultivable species of oral bacteria, particularly Gram negative species, were present in our database. Other published sequences

and about 5 000 sequences available from Ribosomal Database Project [42] and GenBank were also available for comparisons. Similarity matrices were constructed from the aligned sequences by using only those sequence positions for which 90% of strains have data [8]. The similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor [21]. Phylogenetic trees were constructed using the neighbor-joining method of Saitou and Nei [45].

Characterization of the cultured spirochetes using electron microscopy

For ultra-structural analysis using transmission electron microscopy, the cells of strains ADB1, ADB2 and those cultured from the blood of the healthy forester (strain HFB) were harvested by centrifugation and gently suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of about 108 cells per μl. Samples were negatively stained with 1% (Wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 sec. Specimens were examined with a Jeol Model JEM-1200EX transmission electron microscope operating at 100 kV.

Serological analysis

The serum of the healthy forester was analyzed using the Venereal Disease Research Laborartory (VDRL), Rapid Plasma Reagin (PRP) test, Fluorescent Treponemal Antibody Absorption (FTA-ABS), *Treponema Pallidum* Hemagglutination (TPHA), Indirect Immunofluorescent Antibody Test (IFAT) and the Enzyme-Linked Immunoabsorbent Assay (ELISA) tests. In addition, Western blot analysis was also performed (Immunosa, Nyon,CH; and BioGenex Lyme IgG/IgM, D601-Lyme) for the detection of specific anti-*Borrelia burgdorferi* IgG and IgM antibodies. A post mortem serological analysis of the blood and CSF of the AD cases was made using IFAT, ELISA and Western blot (BioGenex Lyme IgG/IgM, D601-Lyme). The serological analyses were made independently in two different laboratories. For the evaluation of Western blot analysis, criteria proposed by the Centers for Disease Control and Prevention (CDC) were applied [6]. Serum of three non-demented cases and the CSF of one non-demented subject were also analyzed. In addition, the blood and CSF of one AD case where Borrelia burgdorferi was not cultivated from the brain was also tested.

Histochemical and immunohistochemical analysis

For characterizing the spirochetes cultivated from the AD brains and from the blood of the healthy forester, as well as detecting spirochetal antigens in brain, the following anti-*Borrelia burgdorferi* antibodies were used at the indicated dilutions: monoclonal anti-OspA (H5332, H3T5, Symbicom, 1:10), Flagellin (G 9724, H605, Symbicom, 1:20), anti-*Borrelia burgdorferi monoclonal* (C63780M, Biodesign,1:30) and polyclonal (Biodesign, B65302R,1:30). Additionally, two rabbit anti-*Borrelia burgdorferi* antibodies prepared in the University Institute of Pathology, CHUV, Lausanne, Switzerland (BB-1017, 1:500 and BB-1018, 1:500) were tested. For the preparation of these polyclonal antibodies, two rabbits (weight 2.5 and 3 kg) were immunized weekly with 0.5 ml of cultured *Borrelia burgdorferi* (strain B31 in BSK II medium) in emulsion with an equal part of Freund's complete adjuvant. They were bled 1 week after receiving the third injection and the sera were used for immunostaining. The specificity of all these mono and polyclonal anti-*Borrelia burgdorferi* antibodies were tested by Western blot analysis (BioGenex Lyme IgG Kit; D601-Lyme), following the instructions of the manufacturer (Fig. 1). For the detection of *Borrelia burgdorferi* specific antigens in the brain of the 3 AD cases, frozen sections were analysed. These were fixed in acetone for 10 minutes at 4°C, pretreated with 1% amylase at 37°C for 3–5 minutes, and washed 3×5 minutes with PBS before use. Two monoclonal antibodies for the detection of bacterial peptidoglycan (Biogenesis 7263-1006 and Chemicon MAB995, 1:100) were also used as previously described in detail (*S9, S10*). In order to determine if spirochetal antigens, bacterial peptidoglycan, and Aβ are co-localized in senile plaques, serial sections, spaced at 14 μm were immunostained with anti-*Borrelia burgdorferi*, anti-bacterial peptidoglycan (Biogenesis 7263-1006 or Chemicon MAB995, 1:200) and anti-Aβ (DAKO, M872, 1:50) antibodies, respectively. For detection, the avidin-biotin-peroxidase technique was used. The sections were incubated with the primary antibody for 24, 48 or 72 hours at 4°C. The immunoreaction was revealed by diaminobenzidine (DAB) alone, or with nickel-ammonium sulfate enhancement. Smears of B31 were used as positive controls. Frozen sections immunostained in the absence of the primary antibody or with an irrelevant mono- or polyclonal antibody were used as controls.

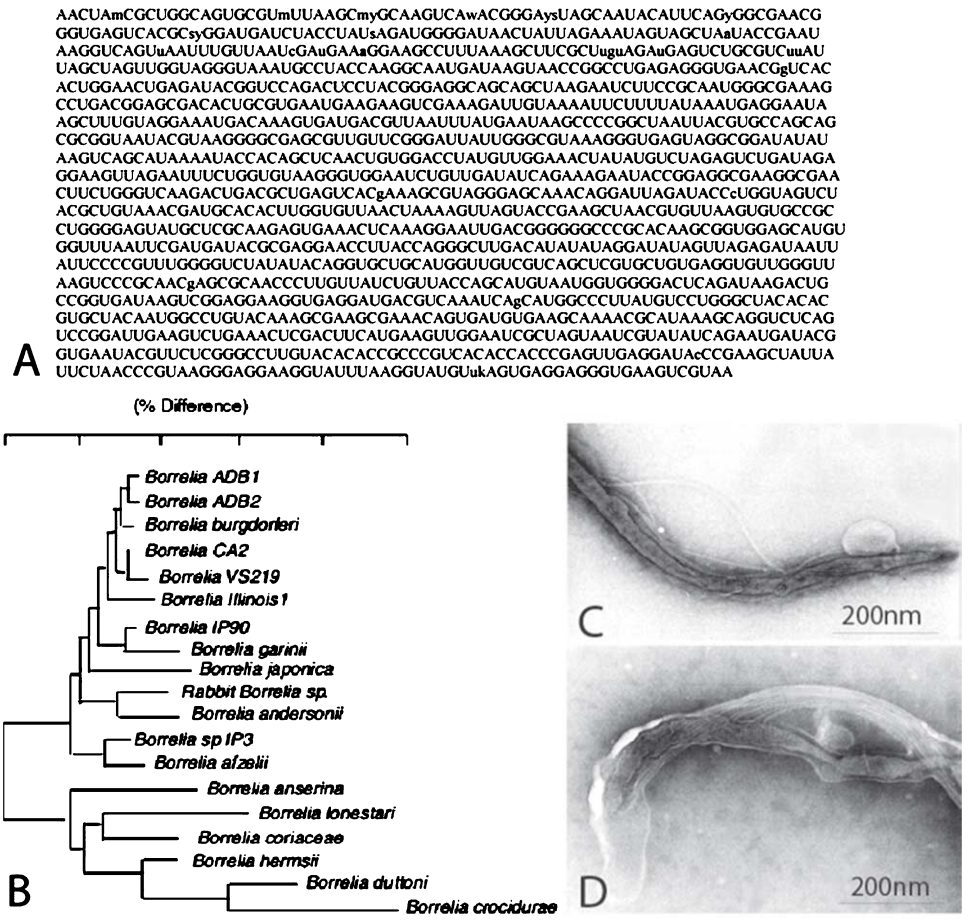

Fig. 1. Phylogenetic and ultrastructural characterization of the spirochetes cultivated from the brain. A and B: The phylogenetic analysis definitely identifies the cultured spirochetes (strains ADB1 and ADB2) cultivated from the AD brain as *Borrelia burgdorferi* sensu stricto. C and D: Strains ADB2 (C) and HFB (healthy forester, D) show the presence of 10–15 periplasmic flagella inserted at each end of the cell, which taxonomically identify them as Borrelia species.

Brain sections of control cases without brain lesions were also used as negative controls.

In situ hybridization

In situ hybridization (ISH) was performed using the Hybaid, OmniGene thermal cycler, equipped with a Satellite Module of *In-Situ* block. For ISH, paraffin sections (5 μm) as well as frozen sections (10 or 20 μm) were utilized. The paraffin sections were dewaxed in xylene, hydrated in 99%, and 95% ethylene and rinsed in pure water 2×3 min. On both frozen and paraffin sections, endogenous peroxidase was blocked by treatment in methanol containing 3% H_2O_2. The sections were treated with 1% hot SDS (70°C) for 5 min, with Lysozyme (25 000 U/ml in PBS, pH 5.5 at 37°C) for 5 min and with Proteinase K (10 μg/ml in 50 mM Tris-HCL, pH 7.6 at 37°C) for 30 min. Following each treatment, the sections were washed in pure water 3×10 minutes. The sections were post-fixed for 20 min with 1% paraformaldehyde in PBS containing 50 mM $MgCl_2$, rinsed with three changes of pure water, and dried in a series of ethanol washes. The sections were incubated with a prehybridization solution (1 μl 0.5M Tris HCl, pH 7.4, 50 μl 20-X- SSC, 1 μl 0.05 M EDTA, 100 μl of 50% dextran sulfate, 250 μl formamide and 98 μl of pure water for a total volume of 500 μl) in the humidity chamber of the thermal cycler at 42°C for 1 hour. The prehybridization solution was then replaced by the hybridization solution containing 100 ng of probe labeled by nick-translation with Digoxigenin (OspA gene BBB012, SN3, position 360–426); flagellin gene BBB032, WK3, position

396–425 purchased from GENSET). The nucleotide sequence of the probes was: 5′–CAA TGG ATC TGG AGT ACT TGA AGG GGT AAA AGC T–3′ and 5′-AAT GCA CAT GTT ATC AAA CAA ATC TGC TTC–3′, respectively. The sections were coversliped, and 10 min incubation at 100°C was followed by an overnight incubation at 42°C in the humidity chamber of the Hybaid cycler. Posthybridization washes were in an equal mixture of formamide and 2-X-SSC, pH 7 at 42°C for 2 × 20 min and in 0.1-X-SSC, 2 mg $MgCl_2$, 0.1% Triton-X-100 at 60°C for 30 min. After a rinse in TBS 3 × 5 min, the sections were treated with a blocking solution containing normal rabbit serum diluted 1:5, 3% bovine serum albumin and 0.1% Triton-X-100 in TBS for 1 hour. For the detection of the hybridization products anti-digoxigenin alkaline phosphatase or peroxidase conjugates were used. The alkaline phosphatase substrate solution or DAB were used as chromogens for visualization of the reaction products. Control sections without specific probes and sections from patients without brain pathology were used as negative controls.

RESULTS

Table 1 summarizes the main results obtained in the present study. Of the three neuropathologically confirmed AD cases, Apo-E genotyping revealed that AD1 and AD2 were 3/4 while AD3 was 3/3.

Characterization of the cultivated microorganisms

For genomic characterization, the full sequences of the 16S rRNA gene for three of the cultivated spirochetes were determined: for strains ADB1 and ADB2 (cases AD1, AD2) and HFB (healthy forester). Although the spirochetal strain ADB1 was contaminated with an unknown bacterium, the use of spirochetal selective primers for PCR enabled genetic analysis of the spirochete to be determined. The sequence of the 16S rRNA gene was identical for the three spirochete strains analyzed as is illustrated in Fig. 1A.

The phylogenetic analysis of the 16S rRNA gene sequence revealed that the cultured spirochetes (strains ADB1, ADB2 and HFB) correspond to *Borrelia burgdorferi sensu stricto (s. s.)*. The phylogenetic position of these spirochetes among other species of spirochetes and borrelial strains is shown in Fig. 1B.

The ultrastructural analysis of the cultured spirochetes (strains ADB2 and HFB) demonstrated that they had ultrastructural characteristics of *Borrelia burgdorferi* species, i.e. thin helical cells with 10–15 periplasmic flagella inserted at each end of the cell (Fig. 1C, D).

Serological analysis

The results of the serological analyses are illustrated in Table 2 and Fig. 2. The analysis and the interpretation of the serological results were made following criteria of the Center for Disease Control (CDC) [6]. A positive serology for *Borrelia burgdorferi* was detected in 2 AD cases (AD 1 and AD3). In case AD3, in addition to a positive Lyme IgG, a positive IgM response was also observed by Western blot, a finding that is known to occur in some untreated patients with chronic Lyme disease (Fig. 2B). It is of interest to note that the *Borrelia burgdorferi* specific 31 kDa OspA band was present in all the 3 AD cases, likewise the p39 band despite it being very weak in two cases, whereas the p34 OspB band was absent. Following CDC criteria, in case AD2 we concluded that the serology was negative, but that the detection of OspA and the weak p39 and p24-25 bands by Western blot was noteworthy. The serological tests of the healthy forester showed the following values: VDRL-; TPHA +320 (normal value >80); FTA-Abs

Table 1
Results of the analysis of the involvement of *Borrelia burgdorferi* in the 3 Alzheimer's cases (AD1, AD2, AD3) where spirochetes were cultivated from the brain

BSK-II	Apo-E	Phylogen	Serology	Antigens	ISH
AD1+	3/4	*Borrelia burgdorferi ss*	+	+	+
AD2+	3/4	*Borrelia burgdorferi ss*	–	+	+
AD3+	3/3	0	+	+	+

BSK-II : + = successful cultivation of spirochetes from the brain in BSK-II medium; Apo-E: results of the Apo-E genotyping. Phylogen: results of the phylogenetic analysis of the spirochetes cultivated from the brain. Antigens : + = presence of *Borrelia burgdorferi* antigens in the brain; ISH = *In situ* hybridization. + = positive; – = negative; 0 = not done.

TP-; IFAT +/– 1/128 (normal value >120) and ELISA +/– 121U (normal value >120). The Western Blot was positive following the results obtained by Immunosa (Nyon, Switzerland) and also following the results obtained employing the BioGenex Lyme IgG Western blot Kit. The Western blot of the serum and CSF of the non-demented controls and of the AD subject where spirochetes were not cultured from the brain, were negative.

Table 2
Results of the serological analysis of blood and cerebrospinal fluid (CSF) of the 3 AD cases and of the healthy forester

	IFAT	ELISA	Western Blot	
			IgG	IgM
AD1 Blood	1/2048 (+)	200U (+)	+	–
AD1 CSF	1/2600 (+)	236U (+)	+	–
AD2 Blood	1/16 (–)	83U (–)	–	–
AD2 CSF	1/16 (–)	84U (–)	–	–
AD3 Blood	0	0	+	+
AD3 CSF	0	224U (+)	+	+
HFB Blood	1/128 (+/–)	121 (+/–)	+*	+*

IFAT = Indirect Immunofluorescent Antibody Test, ELISA = Enzyme-Linked Immunoabsorbent Assay: + = positive; – = negative; 0 = the analysis was not performed. In the case indicated by asterisk the Western blot was performed in parallel by the BioGenex Lyme IgG Western blot Kit and by Immunosa (Nyon, Switzerland).

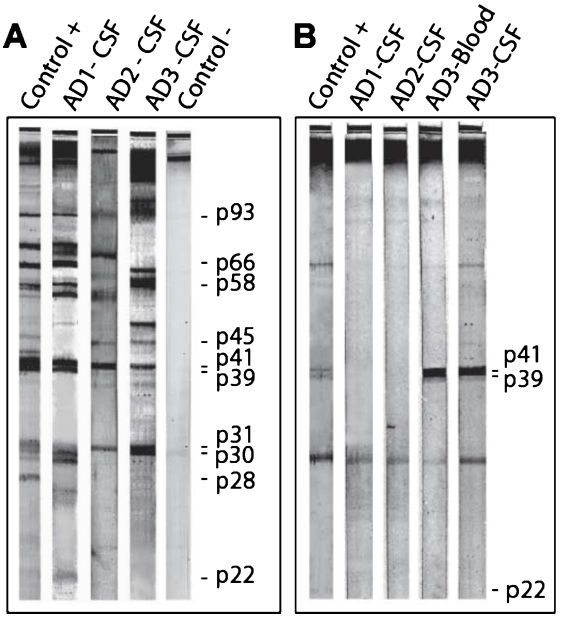

Fig. 2. Western blot analysis of specific anti-*Borrelia burgdorferi* antibodies in the cerebrospinal fluid (CSF) of the 3 AD cases (AD1, AD2 and AD3) using the BioGenex Lyme Western blot kit. Control + = positive control for IgG and IgM was provided by the manufacturer. Control - = control patient without dementia. A: Western blot analysis of specific anti-Borrelia burgdorferi IgG: AD1: p93, p66, p58, p41, p39, p31/30, p22 (positive); AD2: p93, p45, p41, p 31, p25 (negative). Notice a very weak p 39. AD3: p93, p58, p45, p41, p39, p31/30 (positive); Control -: weak p93 and p30 bands (negative). B: Western blot analysis of specific anti-Borrelia burgdorferi IgM: AD1: negative, AD2: negative, AD3: p39, p41 (positive).

Detection of Borrelia antigens and genes in the brain

In the 3 AD cases, cortical atrophy, dissemination of microorganisms in the cerebral cortex in the form of scattered circumscribed colonies, and distribution of beta amyloid deposits were morphologically *similar to previously described* pathological changes in dementia paralytica [39, 19, 24] caused by *Treponema pallidum* (Fig. 3). Thread-like structures disseminated in the cortical neuropil, compatible with individual spirochetes, were also observed.

An immunohistochemical analysis was performed for the detection of Borrelia burgdorferi antigens in the brain of the patients from which Borrelia spirochetes were cultivated. Western blot analysis of 8 different antibodies showed their ability to recognize Borrelia burgdorferi antigens (Fig. 4). The

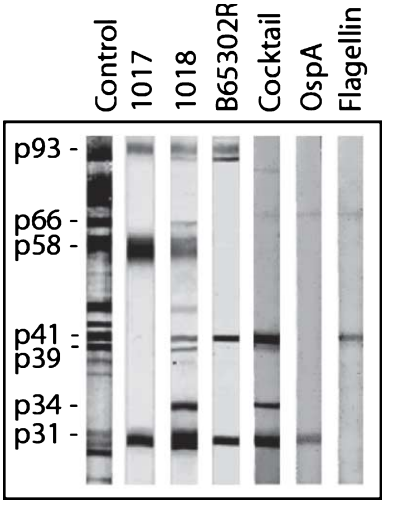

Fig. 3. Western blot analysis of the specificity of anti-*Borrelia burgdorferi* antibodies used in the present study. A: Western blot analysis of the polyclonal anti-*Borrelia burgdorferi* antibodies. Control = positive control provided by the manufacturer (Biogenex Lyme IgG Kit). Te rabbit anti-Borrelia antibodies 1017 and 1018 were prepared in the University Institute of Pathology, Lausanne, Switzerland, B65302 antibody is from (Biodesign). A monoclonal antibody against OspA and Flagellin but also a cocktail of OspA, OspB and Flagellin monoclonal antibodies were also used in the present study.

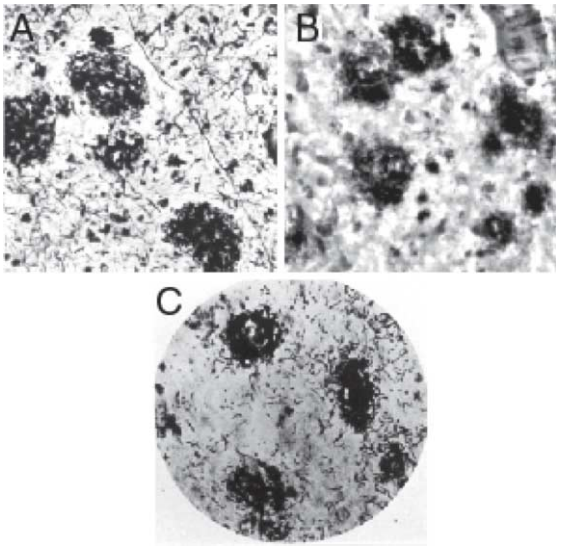

Fig. 4. Illustration of the striking similarity of the agglomeration of spirochetes in the cerebral cortex in case AD1 with positive Lyme serology and in general paresis. Compare the similarity of the silver impregnation pattern when using a modified Bielschowsky stain for senile plaques (A) or using a silver impregnation technique for spirochetes (Warthin and Starry) (B) in the cerebral cortex of case AD1 and in a case of general paresis (C). The permission for reproduction of Figure C was kindly provided by Springer-Verlag publisher and corresponds to Fig. 4 of Jahnel (Abb. 4. 1929)[28].

colony-like masses and part of the disseminated individual filaments showed positive immunoreactions with anti-*Borrelia burgdorferi* antibodies (Fig. 5B), including the anti-OspA antibody. The spirochete antigens showed the same pattern of distribution as amyloid beta peptide (Aβ). Although the immunoreaction was weaker for OspA, the labeling was consistent and was stronger in the center of the colony- or plaque-like structures. *Borrelia burgdorferi* antigens, including OspA were also detected in a number of neurofibrillary tangles (Fig. 5D, E) and in the wall of some blood vessels containing amyloid deposition (Fig. 5F). On serial sections, Borrelia antigens, bacterial peptidoglycan and Aβ were co-localized in senile plaques and in blood vessels.

Borrelia burgdorferi OspA and flagellin genes were also detected in senile plaques and in a number of neurofibrillary tangles in all three AD cases by *in situ* hybridization (ISH) (Fig. 5H). The pattern of distribution was similar to Borrelia antigens. The extranuclear localization of the ISH product excluded the possibility of unspecific DNA labelling. Control sections where the specific Borrelia antibodies or probes were omitted were negative (Fig. 5I).

DISCUSSION

Spirochetes were successfully cultured from the post mortem brains of 3 AD cases and from the blood of a clinically asymptomatic forester. In the present study, 16S rRNA gene sequence analysis identified the spirochetes cultivated from the brain of two AD cases and from the blood of the healthy forester as Borrelia burgdorferi sensu stricto (s.s.). The detection of Borrelia *burgdorferi* specific antigens and genes in the brains of these patients provided further evidence that they suffered from chronic Lyme neuroborreliosis. Consistent with the present findings, the genospecies *Borrelia garinii* and *Borrelia burgdorferi s.s.* have been reported to be predominantly involved in neuroborreliosis [47].

Lyme disease is geographically confined and the incidence is low when compared to AD [5]. This, coupled with the fact that our cases came from a geographic area known to be endemic for Lyme disease, may explain why previous investigators have failed to detect any association of Borrelia with AD [15, 26, 28]. In order to study the particular involvement of Borrelia burgdorferi in AD, it is important to analyze AD patients with a positive serology for Borrelia burgdorferi. Different types of spirochetes may be similarly involved in other AD cases [29, 44]. Antibodies to various spirochetes are highly prevalent in the population at large, and it is important to consider that spirochetes of the oral cavity as well as intestinal spirochetes could contain amyloidogenic proteins and play a role in chronic neuroinflammation. For the majority of these spirochetes, diagnostic and serological tests are not available. In our initial analysis of the potential involvement of spirochetes in AD, we visualized by dark field microscopy helically shaped microorganisms in the CSF, blood and cerebral cortex in 14 AD cases that were absent in 13 control cases [29]. Further analyses using scanning electron-microscopy and atomic force microscopy showed that they possess axial filaments, therefore taxonomically they belong to the order Spirochaetales [30]. Subsequently Riviere et al., using species-specific PCR and monoclonal antibodies, detected oral Treponema in 14/16 AD cases and 4/18 non-AD controls [44]. In endemic areas of Lyme disease, the wide distribution of other spirochetes (e.g. oral spirochetes), which were found to be associated with AD, may mask a clustering of an association of Borrelia burgdorferi with AD. Careful epidemiological studies will be necessary to analyze this point.

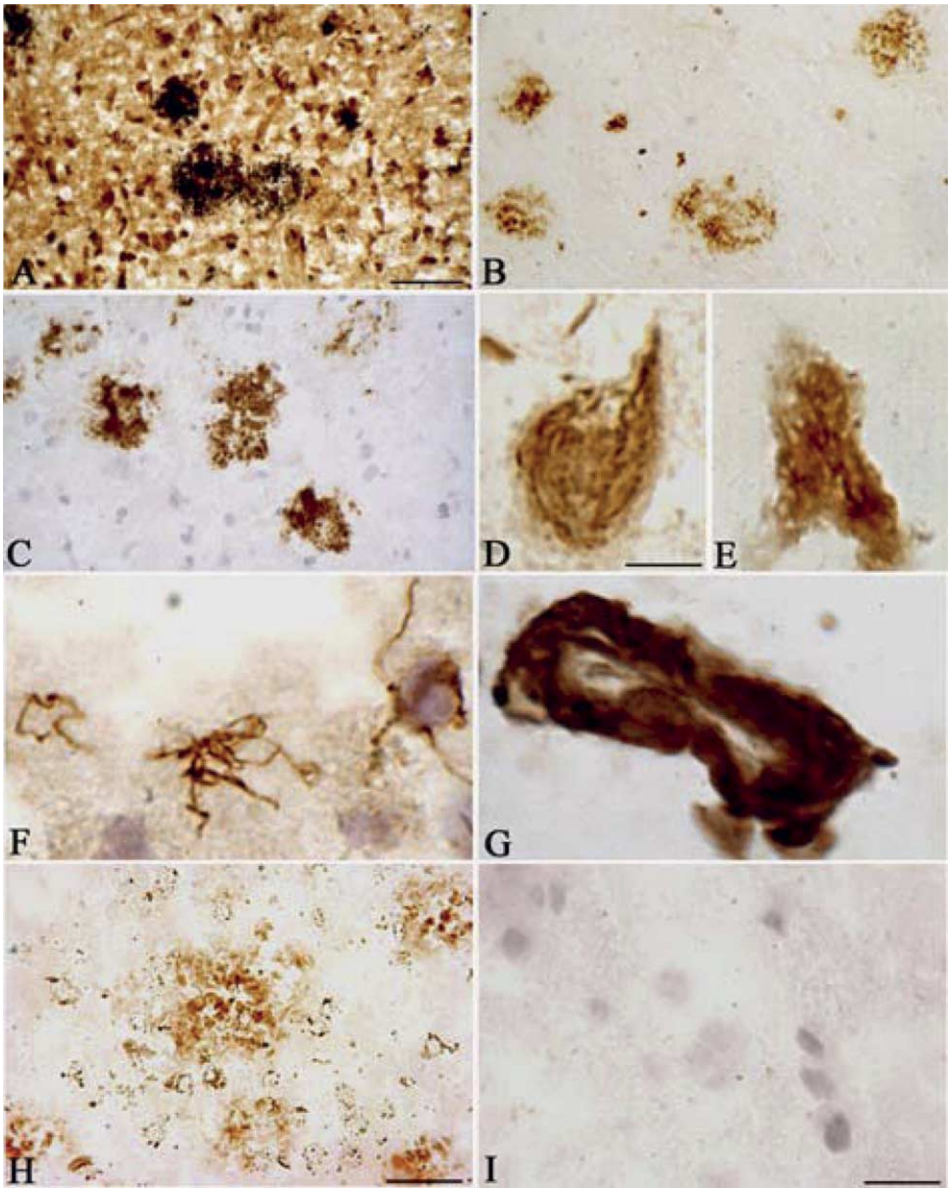

Fig. 5. *Presence of Borrelia burgdorferi* antigens in the brains of the AD cases in which spirochetes were cultivated in BSK II medium. A: The Warthin & Starry silver impregnation technique for spirochetes shows colony like masses of spirochetes in the frontal cortex. B: A similar distribution of Borrelia antigens on adjacent sections as revealed by a rabbit anti-*Borrelia burgdorferi* antibody B65302R, Biogenesis). C: Note the identical distribution of Aβ immunostained senile plaques in the frontal cortex of the same patient. Neurofibrillary tangles were immuno-labeled with rabbit anti Borrelia antibody 1017 (D) and anti-OspA antibody (E). F: Individual spirochetes showing immunoreactivity to anti-Borrelia antibody in the cerebral cortex. G: Leptomeningeal vessel showing positive immunoreaction to bacterial peptidoglycan. H: The pattern of distribution of *Borrelia burgdorferi* genes as detected by *in situ* hybridization was identical to those of Borrelia antigens and Aβ. Control sections in which the primary antibody (I) or the specific probes were omitted (not shown here) were negative. Scale bar in A is the same for B-C and G = 100 μm; bar in D is the same for E and F = 10 μm; H = 50 μm; I = 20 μm.

Based on previous analyses we also suggested that amyloidogenic protein may be integral part of spirochetes [29, 33]. These observations were reinforced by Ohnishi et al. [40] who showed that the outer surface protein (OspA) of *Borrelia burgdorferi* forms amyloid fibrils *in vitro*, similar to human amyloidosis.

Reports of associations between infection and AD are not confined to spirochetes. The presence of

Herpes virus type 1 (HSV-1) in the AD brain has been reported [20, 17, 18]. Chlamydia pneumoniae was also found to be associated with AD. Mice exposed to Chlamydia developed AD-like amyloid plaques [2, 23].

The pathological findings observed in the 3 AD cases were reminiscent of those described as long ago as 1929 in dementia paralytica caused by Treponema pallidum (see Fig. 5). They are consistent with primary parenchymatous involvement of tertiary Lyme neuroborreliosis. Similar to the observations of Noguchi and Moore with respect to Treponema pallidum [38], our results show that Borrelia burgdorferi may also persist in the brain in chronic Lyme neuroborreliosis and be associated with cortical atrophy, amyloid deposition, and clinical dementia. The present findings reinforce the similarity of clinical and pathological manifestations of syphilis and Lyme disease and suggest that *Borrelia burgdorferi* may also be involved in the pathogenesis of several chronic neuro-psychiatric disorders. The case of the healthy forester, where the 16s rRNA analysis also defined the spirochetes cultivated from the blood as Borrelia burgdorferi s.s., indicates that, it could represent an acute, asymptomatic infection or may correspond to a more chronic latent stage of the disease. A clinical follow-up and repeated serological tests and cultures would be necessary to answer this question.

A well-defined risk factor for late onset AD is the epsilon-4 variant of the apolipoprotein E gene. The Apo-E genotyping of the three AD cases suffering from Lyme neuroborreliosis, showed that two of them possessed the epsilon-4 allele. The low number of cases does not allow any conclusive evidence, however this result may suggest that patients with genetic risk factors, such as carriers of the epsilon-4 allele of Apo-E, or promoter polymorphisms in pro-inflammatory cytokines, infection may result in a more severe phenotype, which includes enhanced Aβ accumulation when compared to non-carriers. The clinical and pathological hallmarks of Alzheimer's disease (AD) are present in the atrophic form of general paresis [19, 24, 39]. In general paresis the accumulation of "plaques" in the cerebral cortex, the cortical atrophy and the amyloid deposition, as generally accepted, are secondary to the spirochetal infection. Similarly, in several other chronic bacterial infections or in experimental amyloidosis the bacterial infection or bacterial exposure always precede the amyloid deposition. In patients with genetic defect which facilitate infection the genetic problem will be the first step in the cascade of events, followed by infection than with amyloid deposition.

The results of this multifaceted study allow us to conclude that *Borrelia burgdorferi,* like *Treponema pallidum* in syphilis, may persist in the brain and is associated with amyloid plaques in AD. The data suggest that Borrelia burgdorferi, perhaps in an analogous fashion to Treponema pallidum, may contribute to dementia, cortical atrophy and amyloid deposition.

In vitro and *in vivo* analyses exposing mammalian CNS cells or experimental animals to Borrrelia burgdorfgeri or other cultivatable spirochetes will bring further information about a potential causal role of spirochetes in amyloidogenesis. The AβPP transgenic mouse expressing mutant human AβPP is a well recognized model for AD, albeit incomplete. Future studies, where such animals are infected with spirochetes, may also give further insight into their significance in contributing to the pathogenesis of AD.

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Statistical Evidence for a Lack of Correlation between the Incidence of Lyme Disease and Deaths Due to Alzheimer's Disease

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Abstract. Reports that Lyme disease (LD) causes Alzheimer's disease (AD) have appeared in academic journals and online. If the biological agent *Borrelia burgdorferi* that causes LD also causes AD then areas with highest levels of LD should have significantly higher numbers of deaths due to AD compared to low LD areas. Here we show there is no statistically significant correlation between the incidence of LD and deaths due to AD in the US. Furthermore, the 13 states with the highest deaths due to AD were statistically different ($P < 0.0001$) from those with high LD incidence. Recent work by several other research groups has validated this conclusion.

Keywords: Lyme disease, Alzheimer's disease, *Borrelia burgdorferi*, disease incidence

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease with no proven initiating cause. Currently over 5,000,000 individuals in the US have the disease which is the sixth major cause of death [1]. Understanding the causes of Alzheimer's is a medical priority. Lyme disease (LD), which leads to the neurodegenerative disease neuroborreliosis, is caused by the bacterium *Borrelia burgdorferi* [2]. Independent research groups have presented different lines of evidence arguing that LD can cause AD [3–6]. Thus there are valid reasons to believe that LD could cause AD. *B. burgdorferi* is a member of the spirochete family of bacteria and spirochetes such as *Treponema pallidum* can cause dementia [7]. A diversity of post-mortem techniques (e.g., ultrastructural visualization, immunolocalization, western blot, RNA sequence analysis, ELISA, PCR, etc.) have revealed suggested the presence of various spirochetes, including *Borrelia burgdorferi*, in the brains of AD individuals [3–5, 8]. The relationship between LD and AD has also been supported by analysis of existing data using Koch's and Hill's Postulates [8]. What is lacking to fulfill these postulates is the isolation of functional entities (e.g., *Borrelia burgdorferi*) followed by proof of their ability to induce AD-related events in tissue culture cells.

Two hallmarks of Alzheimer's are the build-up of plaques and tangles in the brain, which are linked to neurodegenerative events [9, 10]. *Borrelia burgdorferi* can induce both amyloid beta and phosphorylated tau formation in tissue culture cells [5]. Thus accumulating circumstantial evidence does suggest a link between LD and AD. A quick internet search reveals that this link has spawned widespread concern (e.g. [11, 12]). The ongoing interest and concern makes

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it critical to validate or disprove any such relationship between the two diseases. Here we test a simple hypothesis: If LD does cause AD then the incidence of deaths due to AD should higher where LD is most prevalent compared to low LD areas.

MATERIALS AND METHODS

To our knowledge, location-specific data for the reporting of cases of both LD and deaths by AD is only available for the US. The age-adjusted death rates for AD were taken from Fig. 4 in Tejada (2013). The data for the reported cases of LD by state (2002–2011) were from the Centers of Disease Control and Prevention website (June 25, 2013 [14]). Although many factors affect the accuracy of the disease including the consistency of reporting of LD and deaths AD, which will varies between states, it is assumed that this variability is negated by the comparison of 13 different states for each disease. The data sets used were the best and most current available at the time of writing. Means and standard deviations were calculated for sets of 13 states: those with the highest incidence of LD (18.978–68.222 cases/1000) and those with low to non-existent (0.0–0.189) LD. The Mann-Whitney U test was used to determine if AD is more prevalent in states with high LD compared to those with low LD. AD incidence in the 13 states with the highest LD incidence (MN, MD, VT, NY, WI, RI, ME, PA, NJ, MA, NH, DE, and CT) was compared with AD incidence in the 13 states with the lowest LD incidence (CO, AR, OK, LA, AZ, MS, NM, UT, GA, AL, SD, MT, and WA). Hawaii which has a zero incidence of LD was excluded because it is a distinct entity outside of the contiguous US states. Fisher's exact test was used to determine if the 13 states with the highest AD incidence (LA, VT, CO, AL, MS, IA, KY, SC, AZ, SD, ND, TN, and WA) were also states with high LD incidence. Fisher's exact test was also used to determine if high AD was correlated with high LD in a two-by-two contingency table (Table 1).

RESULTS

The mean value for the incidence of LD was calculated for each state and plotted beside Alzheimer's death rates (Fig. 1). With the exception of Vermont the 13 highest LD states are different from the 13 highest AD states. In fact, 7 of the 13 highest LD states fall within the 13 states with the lowest incidence of deaths due to AD. We tested these observations against the assumption that if LD and AD were correlated, they should share most if not all of the high incidence states in common. The results of the statistical analysis reveal the 13 highest AD states are different from the 13 highest LD states ($P < 0.0001$). To determine if there is any correlation between AD and LD, the annual incidence of deaths due to AD were compared between U.S. states which had the highest and lowest incidences of LD. Although AD incidence was slightly greater in states with low LD this difference was not significant. Therefore AD is not more prevalent in high LD states (Fig. 2). Taken one step further, Table 1 presents a two-by-two contingency table of high and low AD states versus high and low LD states. Fisher's exact test resulted in a two-tailed P-value of 0.015 indicating a correlation between either low AD and high LD and/or high AD and low LD. This means high AD is not correlated with high LD.

Table 1
Two-by-two contingency table of the states with high and low AD levels that also exhibit either high or low LD levels; From [19]

	High LD	Low LD
High AD	1	7
Low AD	7	2

Fisher's exact test two-tailed P-value = 0.015. This statistically significant difference indicates a correlation between either high AD and low LD and/or low AD and high LD, demonstrating that high AD is not correlated with high LD.

DISCUSSION

If *Borrelia burgdorferi* causes AD, then areas with the highest incidence of LD should also have significantly higher numbers of deaths due to AD. A simple graphic presentation of the incidence of LD state-by-state in the USA versus the incidence of deaths due to AD clearly reveals a lack of co-incidence between these two variables. When deaths due to AD in 13 of the states with the highest incidence of LD were compared with those with the lowest, there is no statistically significant difference between them. Furthermore, if LD and AD are correlated, they would have most of the high incidence states in common. However, the 13 highest AD states are significantly different ($P < 0.0001$) from the 13 highest LD states. Moreover, Table 1 demonstrates not only a lack of correlation but rather an association between high AD and low LD suggesting one disease excludes the other. However due to the small

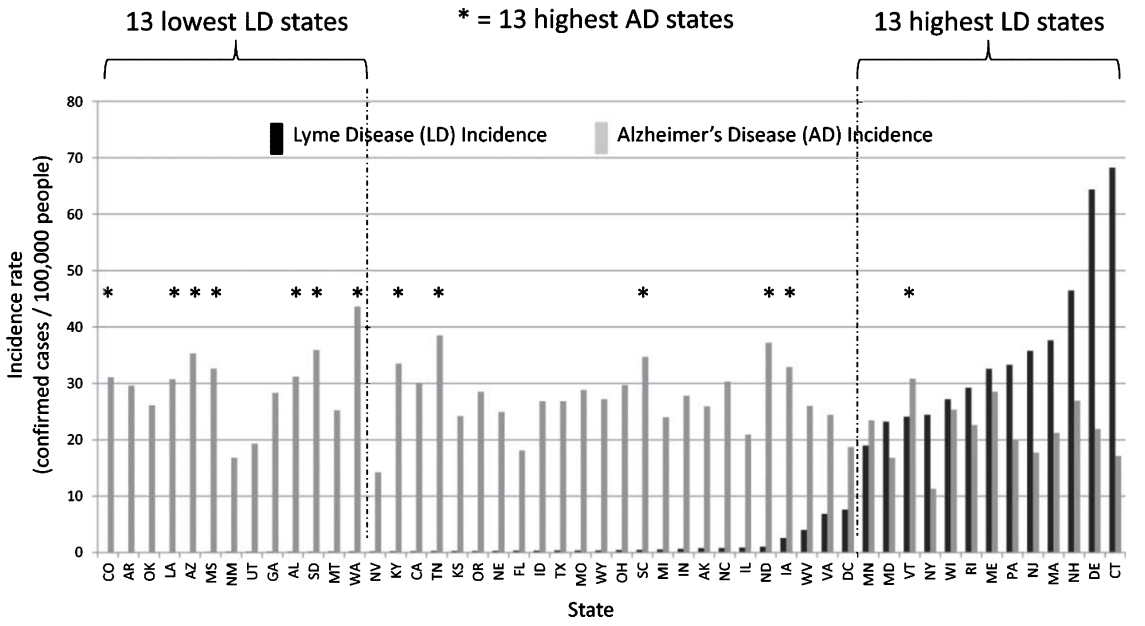

Fig. 1. Incidence of Lyme disease (LD, black) and of deaths caused by Alzheimer's disease (AD, grey) by U.S. state. States are ordered by increasing LD rate. The 13 highest and lowest LD rates (brackets) and 13 highest AD rates (*) are indicated; From [19].

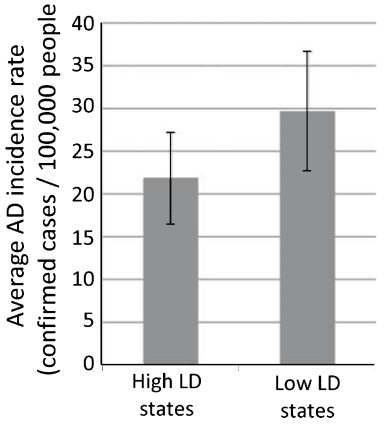

Fig. 2. Deaths by Alzheimer's disease (AD) rate in high versus low Lyme disease (LD) states; From [19].

numbers we interpret the data as simply showing no relationship between the two diseases. The lack of correlation between the incidence of LD and AD on a state-by-state basis and the lack of any demonstrated relationship between the two diseases argue there is no link between them. In support of this, Krut et al. [15] examined the levels of Alzheimer's-specific biomarkers in cerebrospinal fluid (CSF) revealing that the neuroborreliosis profiles were different from the AD profiles suggesting some of the early correlative studies between the two diseases may not be accurate.

It is important to recognize that LD and AD are two distinct diseases with different latency periods. Although Lyme disease has a predominant effect on young males aged 5–9, the disease significantly affects individuals of all ages [16]. While it might seem that a disease affecting young people might not translate to an effect in aging individuals, recent research has revealed that the cause(s) of AD occur decades before any symptoms of the disease manifest themselves [10].

The authors have used the best data available to evaluate the proposed link between Lyme disease and Alzheimer's disease. This does not mean that these data are without issues including consistency of data collection between states and the two diseases, among other variables. What the data do reveal is that there is no evidence to argue that these two diseases are in any way related which hopefully will be communicated to those who have concerns about the relationship between LD and AD. This conclusion has been supported by others.

A recent report by Phillip J. Baker, Ph.D., who is the Executive Director of the American Lyme Disease Foundation, has validated this conclusion using different statistical methodology and more recent data [16]. His linear regression analysis is shown in Fig. 3. To quote Dr. Baker, "An analysis of the data by linear regression analysis generates a correlation coefficient of 0.0753 ($t = 0.262$ for 12 degrees of

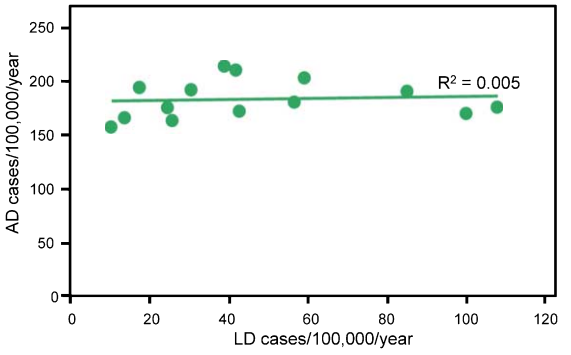

Fig. 3. Linear plot of data for confirmed cases of Lyme disease (2013) and Alzheimer's disease (2014) for States with >95% of all reported cases of Lyme disease in the U.S. Redrawn from Baker [16].

freedom; $p > 0.05$. This indicates no direct relationship between the incidence of Lyme disease and deaths due to Alzheimer's disease in States that account for >95% of all reported cases of Lyme disease in the U.S."

In support of this, Forrester et al showed the "absence of a positive correlation between the geographic distribution of Lyme disease and the distribution of deaths due to Alzheimer disease, ALS, MS and Parkinson disease provides further evidence that Lyme disease is not associated with the development of these neurodegenerative conditions" [17]. Thus three independent studies by different research groups have provided data showing that Lyme disease is not a cause of Alzheimer's disease.

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Alzheimer's Disease: Assessing the Role of Spirochetes, Biofilms, the Immune System, and Amyloid-β with Regard to Potential Treatment and Prevention

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Abstract. Alzheimer's disease (AD) is an infectious disease caused by spirochetes, and these spirochetes form biofilms, which attract the innate immune system. The innate immune system first responder, Toll-like receptor 2, generates both NF-κB and TNF-α which try to kill the spirochetes in the biofilm, but cannot penetrate the "slime". NF-κB is also responsible for the generation of amyloid-β (Aβ) which itself is anti-microbial. Aβ cannot penetrate the biofilm either, and its accumulation leads to destruction of the cerebral neurocircuitry. Treatment with penicillin (as in tertiary syphilis, the comparator to AD) is outlined; a biofilm dispersing agent may need to be added to the protocol.

Keywords: Amyloid-β, biofilm, innate immunity, spirochetes, treatment

Where spirochetes have been found in the brains of Alzheimer's disease (AD), it may be considered an infectious disease; this is the first and most important consideration [1, 2]. It is also a chronic disease, a biofilm-associated disease, [3] and an autoimmune disease [4]. Further, it is a debilitating disease, a socially-destructive disease, an exceedingly expensive disease, and, lastly, a deadly disease [5]. This review will focus on the biofilm portion of the disorder as well as the autoimmune response. It will also touch on some rational therapeutic concepts for this most irrational of diseases.

The infectious nature of AD was revealed when spirochetes (both dental and Lyme) were shown to be present in the brains of affected patients [1]. The dental microbes travel from the oral cavity during times of disruption of the dental plaque and subsequent bacteremia following dental procedures; i.e., any time blood is seen. The hippocampus (which is the initial site of cerebral involvement in AD) is approximately 4 cm from the posterior pharynx. Lyme borrelia travel to the brain via the blood stream during the secondary stage of that disease following the erythema migrans lesion [6]. This secondary stage is characterized by fever, myalgias, arthralgias, and other systemic symptoms. The spirochetes have an affinity for neural tissue and pass through the blood-brain barrier easily [7].

Once the spirochetes are in the brain, they attach, divide (albeit very, very slowly) [8], and multiply. When they reach a quorum, they begin to spin out a biofilm (Fig. 1) [9]. This represents approximately 150 spirochetal cells which are 0.3 microns in

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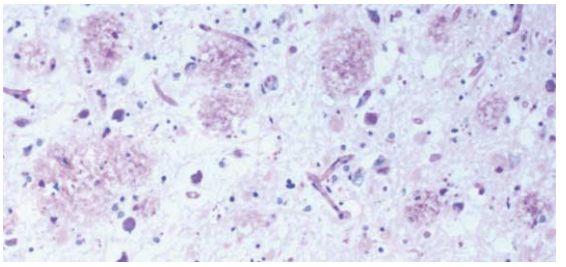

Fig. 1. Hippocampal plaques consisting of biofilms. AD brain plaques: polysaccharides of biofilms stain pink with PAS. PAS 10X. From Allen et al. [8].

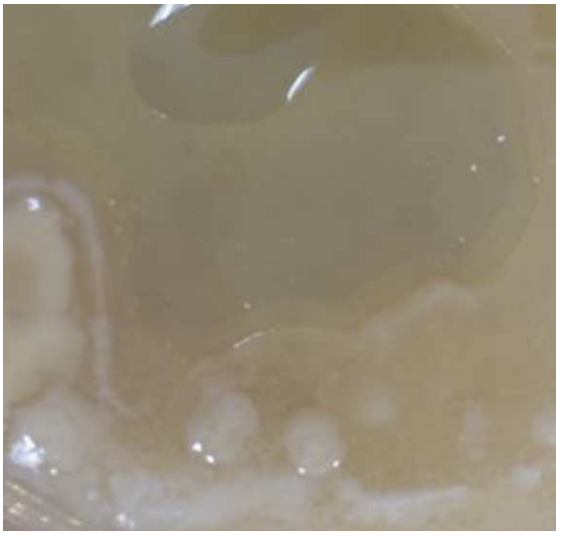

Fig. 2. Representative biofilm (slime). "Slime" represents typical biofilm on gross examination. From Allen et al. [8].

diameter (10 cells are necessary on a two-dimensional culture plate for a quorum to begin). Because of the exceedingly slow division, it takes approximately 2 years to accumulate sufficient organisms to make one biofilm. The biofilm is protective and is a response of the organisms to ensure their survival, inasmuch as it encases them in "slime" (Fig. 2).

Quorum sensing is one triggering mechanism for the production of biofilms; other organisms in other diseases may form biofilms when subjected to different stimuli. These stimuli include salt and water, as seen in eczema and tinea versicolor [10, 11]. Low dose antibiotics and quorum sensing are seen in psoriasis [12] and arthritis [4]. Further, elevated temperatures and exposure to alcohol and other chemicals promote biofilms [13].

At some point after attachment and formation of the biofilms, the innate immune system becomes

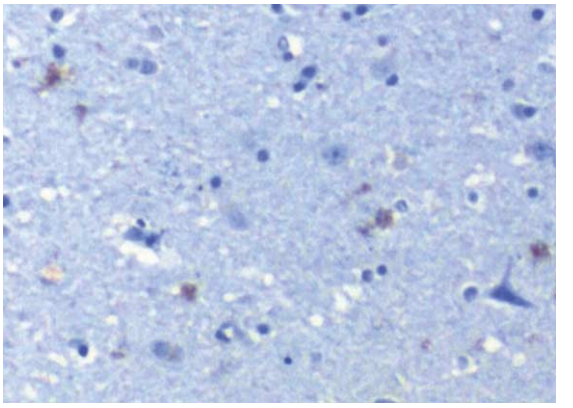

Fig. 3. TLR 2 in hippocampus of AD patient. TLR 2 (CD 282) stains brown (represents activation) 10X. From Allen et al. [8].

activated and attempts to destroy them [8]. Even though the spirochetes are weakly gram negative, Toll-like receptor 2 (TLR 2) has been shown to be the first responder to the organisms incorporated in the extracellular polysaccharide slime (Fig. 3) [8]. TLR 2 itself has recently been shown to be attracted to the "curli" fibers produced by the organisms within the biofilm [14]. These fibers are the major component of the proteinaceous portion of the biomass and are not only immunogenic, but are also important in the attachment of the biofilms. Ordinarily, Toll-like receptor 4, rather than TLR 2, responds to gram-negative organisms.

TLR 2 kills primarily by means of tumor necrosis factor-α (TNF-α) generated by the myeloid differentiation pathway D88 (MyD88). TLR 2 coats the microbes (Fig. 4) and generates both nuclear factor-κB (NF-kB) and TNF-α. This is the process utilized for killing when the organisms are planktonic (free floating) and not in a biofilm. Neither TLR 2 nor TNF-α can penetrate biofilm; consequently, it has been theorized that the TNF-α destroys the surrounding neural tissue instead [8].

Almost all organisms make biofilms. As has been previously stated, these biofilms protect the microbes dwelling within from noxious agents whether chemical, immunologic, or other. The bulk of a biofilm is made up of extracellular polysaccharides. Inside and out there are curli fibers; other amyloid fibers may be within and their purpose is to serve as an infrastructure for the polysaccharides. There are also DNA and water channels, as well as the microbes themselves within the biofilm [15, 16]. None of the commonly used antibiotics penetrate biofilms; and, none of the immunologic molecules from either arm

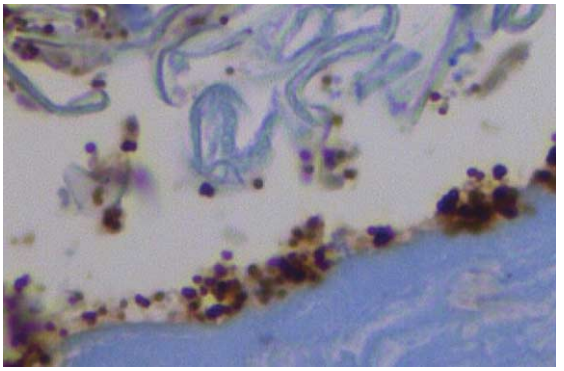

Fig. 4. Candidiasis–TLR 2 coats the yeasts. Activated TLR 2 coats yeasts in the stratum corneum in candidiasis; control location of TLR 2 is in epidermal basal layer (CD 282) 40X. From Allen et al. [8].

of the immune system, whether innate or adaptive, are able to penetrate either.

Ordinarily, the adaptive immune system including B cells, immunoglobulins, and T cells with their cytokines are excluded from the brain by the blood-brain barrier. That is until traumatic brain injury disrupts that barrier: at that point, B lymphocytes and IgG flood the cerebrum [17]. These immunogens kill by complement, alternate complement, killer T cells, cytokines (including TNF-α and others). The killing of brain tissue around the plaques of AD is much more rapid and much more destructive with the adaptive immune system. This is without doubt the reason that AD occurs within 3 years after a cerebrovascular accident; ordinarily, it takes 30-50 years to develop. Further, it is most probably the reason that chronic traumatic encephalopathy (CTE) is so rapidly progressive after many concussions [8]. A concussion may pictorially and practically be considered an ecchymosis, and, as such, is comparable to a hemorrhagic cerebrovascular accident. CTE is currently the scourge of the National Football League where head trauma is a frequent occurrence.

Elucidation of the role of amyloid-β (Aβ) has been challenging: Aβ is a constant in AD and, in fact, it has been thought to be pathogenic by many. It, however, has been shown recently to be antimicrobial [18] and even more recently the pathway to its formation has been made apparent [19, 20] This pathway (Fig. 5) derives from the MyD88 pathway activated by TLR 2. TNF-α, generated by TLR 2, in conjugation with TNF-α converting enzyme (TACE) becomes alpha secretase and splits amyloid precursor protein (APP) to make amyloid alpha. The NF-kB generated by the same MyD88 pathway, together with Aβ converting enzyme (BACE), activates beta and gamma secretases that cleave the APP. The APP then becomes Aβ and attacks the biofilm (Figs. 6, 7) but cannot penetrate it. Consequently, it encompasses the biofilm and its buildup destroys the neurocircuitry of the brain.

This is the very essence of autoimmunity, namely the body attacking itself; this occurs when the body's own innate immune system produces TNF-α or Aβ and attacks the biofilm encasing the spirochetes. In the process of doing this, the surrounding tissue is destroyed instead. Such is the case with the biofilm produced by staphylococcus in eczema and streptococcus in psoriasis; these biofilms call forth the innate immune system and the whole process of tissue destruction is set in motion [4]. The consequences of AD are much more dire however, because they lead to total destruction of the mind.

Any treatment of AD must take into consideration these biofilms. The pathway toward such treatment has previously been set by the treatment of syphilis. Syphilis, in its tertiary form (general paresis of the insane), has been shown to have exactly the same pathology as AD. The same plaques, neurofibrillary tangles, Aβ, and tau protein are present in both.

Where the pathology is the same and where both diseases are caused by spirochetes, and where spirochetes are sensitive to penicillin, a reasonable approach would be to follow the same treatment schedule as syphilis [21]. With that treatment, penicillin administered at any time prior to the onset of tertiary syphilis is curative. The same can reasonably be said for AD; penicillin administered any time prior

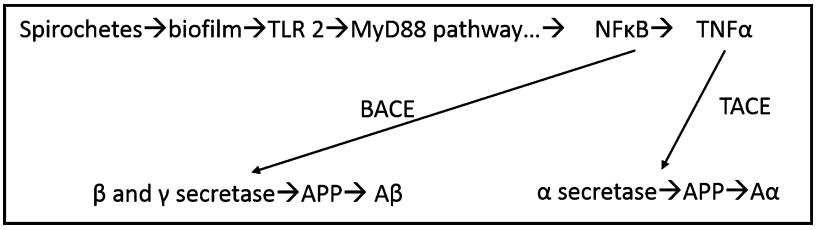

Fig. 5. Possible pathway for development of Aβ. Schematic for production of Aβ and Aα via MyD88 pathway. From Allen et al. [8].

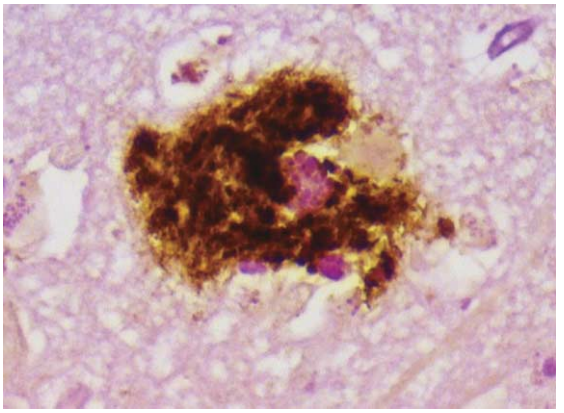

Fig. 6. Aβ co-localizes with PAS (biofilm) in AD plaque. Combined PAS stain and Aβ immunostain; shows co-localization of biofilm and Aβ 40X. From Allen et al. [8].

to the onset of tertiary disease would also be curative. Lyme disease is most closely aligned with syphilis with erythema migrans equivalent to the chancre. In most cases, it is one tick bite compared to one chancre, so the treatment could be reasonably the same [7]. With dental organisms, exposure is ongoing; thus, the treatment would need to be tailored to the patient's dental health. One could imagine penicillin administered once or twice yearly (or perhaps more frequently) in certain situations (CTE?). The same could be said for the 5% of AD "pre"sufferers who have the *APOE ε4* gene for AD. CTE mimics the genetic disease. It must be stated that any neural damage is irreversible; thus, the importance and urgency of treating early in this disease course.

Treatment for patients in the early stages of dementia would need more than penicillin; they would also need an agent to disperse the biofilm [22]. Fortunately, there are such agents, and many are already being employed in AD patients. These agents include furans (citalopram), [23] thiophenes (olanzapine), [24] piperidines (donepezil), [25] pyrroles (azoles), [26] and rifampin [27]. Donepazil, for example, may be an anticholinesterase inhibitor, but it is also a biofilm disperser, so it may be helpful for a short time, but be harmful long term. The dispersal effect would potentially create many more plaques. The same may be said for haloperidol whose use in AD is already shunned.

Specifically, for early dementia, penicillin may be administered as IV or IM injections (IM would be 1.2 mu biweekly for 3 doses), probenecid 500 mg bid (to increase the serum concentration of penicillin by decreasing excretion, citalopram 20 mg daily, and rifampin 500 mg bid. These may be adjusted with the use of other medications. None of this is codified; but, the current treatment is most likely harmful with the biofilms being dispersed without the spirochetes being killed. This would conceivably lead to many more biofilms, because all the spirochetes within the previous biofilm are capable of making new biofilms.

The other major consideration is to treat in the "latent" stage for AD with penicillin by itself. Presumably, this would be similar to the treatment of

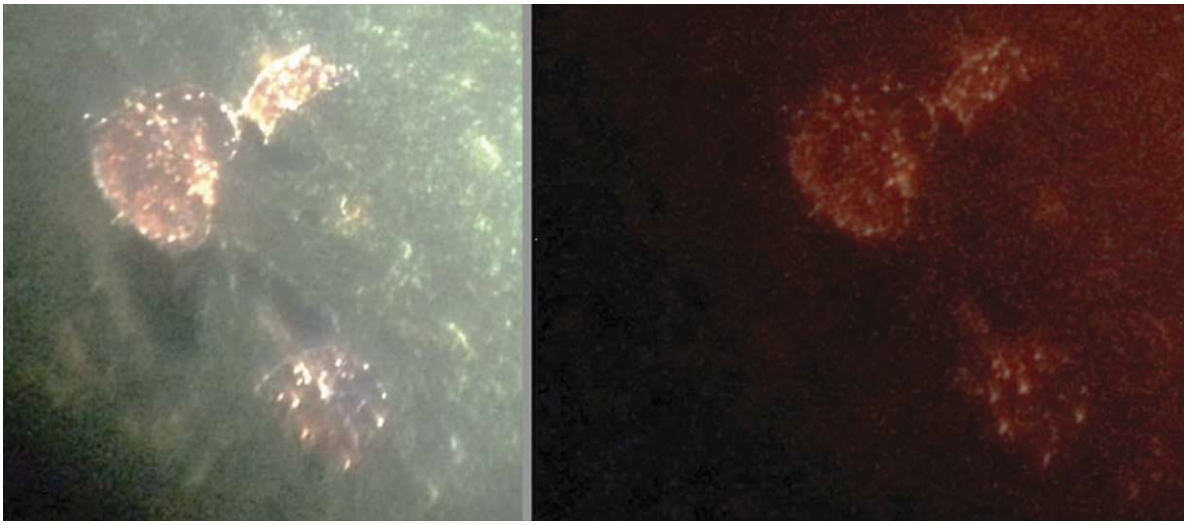

Fig. 7. Same plaques in AD stained for Aβ (left) and biofilm (right). Left Congo red; right, FISH analysis of biofilm (Cy5 red label); shows Aβ and biofilm in exactly the same plaques. Dark areas show water channels, a constant finding in biofilms. Alan MacDonald, M.D., kindly provided this figure.

latent syphilis. Also important would be to treat prior to any dental surgery just as is being done for joint implants. Consequently, the organisms would be treated before they reached the brain in the case of dental surgery and before they did damage (made biofilms) in latent disease. Syphilis, in truth, is different because its presence is revealed by a serology. However, until a serologic test is available for AD, treatment, as has been proposed herein, seems rational. It is also relatively inexpensive, both as to medical costs and the cost of ongoing care of dementia patients.

The story of AD is then one of spirochetes that make biofilms that activate the innate immune system. The first responder is TLR 2 and TLR 2 generates NF-kB and TNF-α that not only damage tissue in an attempt to kill the biofilm-encased spirochetes, but also lead to the production of Aβ. All of the foregoing leads to dementia. Treatment with a bactericidal antibiotic with a concomitant biofilm disperser seems most reasonable; but, as has been stated previously, any neurologic damage is irreversible. It is therefore of the utmost importance to treat early in the course of this disease.

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Bacterial Amyloid and DNA are Important Constituents of Senile Plaques: Further Evidence of the Spirochetal and Biofilm Nature of Senile Plaques

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Abstract. It has long been known that spirochetes form clumps or micro colonies *in vitro* and *in vivo.* Cortical spirochetal colonies in syphilitic dementia were considered as reproductive centers for spirochetes. Historic and recent data demonstrate that senile plaques in Alzheimer's disease (AD) are made up by spirochetes. Spirochetes, are able to form biofilm *in vitro*. Senile plaques are also reported to contain elements of biofilm constituents. We expected that AβPP and Aβ (the main components of senile plaques) also occur in pure spirochetal biofilms, and bacterial DNA (an important component of biofilm) is also present in senile plaques. Histochemical, immunohistochemical, and *in situ* hybridization techniques and the TUNEL assay were used to answer these questions. The results obtained demonstrate that Aβ and DNA, including spirochete-specific DNA, are key components of both pure spirochetal biofilms and senile plaques in AD and confirm the biofilm nature of senile plaques. These results validate previous observations that AβPP and/or an AβPP-like amyloidogenic protein are an integral part of spirochetes, and indicate that bacterial and host derived Aβ are both constituents of senile plaques. DNA fragmentation in senile plaques further confirms their bacterial nature and provides biochemical evidence for spirochetal cell death. Spirochetes evade host defenses, locate intracellularly, form more resistant atypical forms and notably biofilms, which contribute to sustain chronic infection and inflammation and explain the slowly progressive course of dementia in AD. To consider co-infecting microorganisms is equally important, as multi-species biofilms result in a higher resistance to treatments and a more severe dementia.

Keywords: Alzheimer's disease, amyloid, AβPP, amyloid beta, bacteria, biofilm, *Borrelia burgdorferi*, colonies, chronic infection, spirochetes, thioflavin S, Treponema spirochetes

INTRODUCTION

Historic and recent observations demonstrate that senile plaques in Alzheimer's disease (AD) are aggregated masses or colonies of spirochetes identical to those formed by *Treponema pallidum (T. pallidum)* and *Borrelia burgdorferi* (*B. burgdorferi*) in syphilitic and Lyme dementia [1–5]. Various types of spirochetes of the order Spirochaetales [1, 4, 6, 7], including *B. burgdorferi* [1, 2, 8, 9] and several periodontal pathogen spirochetes (*T. denticola, T. socranskii, T. pectinovorum, T. amylovorum, T. maltophilum, and T. medium)* were detected and/or cultivated from the AD brain [4, 7, 10]. Spirochetes persist in the affected host tissues and establish chronic infection and inflammation and are directly responsible for the late or chronic manifestations of

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various spirochetoses, including Lyme disease [2, 3, 7, 11].

It has long been known that spirochetes form aggregated masses or colonies *in vitro* and *in vivo.* Following Steiner, the formation of cortical spirochetal colonies in general paresis is a form of resistance to adverse conditions and a source of reproduction under more favorable conditions [12]. Spirochetal colony formation of various *Treponema* and *Borrelia* species has been the subject of further investigations during the last decades [13–15]. Spirochetal colony formation also occurs in primary cell and organotypic cultures exposed to *B. burgdorferi* [15, 16]. These *in vitro* formed spirochetal colonies showed morphological and biochemical similarities to senile plaques and were immunoreactive to amyloid beta (Aβ), an important component of senile plaques.

Most microorganisms have the ability to form biofilms. Bacteria in biofilm are covered by a "slime"-layer, which protects them from stressful environmental conditions [17–19], therefore, the cultivation and eradication of microorganisms in biofilms is more difficult [20]. Recently, Sapi and collaborators reported evidence that *B. burgdorferi* is able to form biofilms *in vitro*. They also observed *Borrelia* biofilms in skin and lymphocytoma in patients with Lyme disease [21–23]. Biofilm formation in joints in osteoarthritis was also reported [24]. In these diseases, oral spirochetes and *B. burgdorferi* are implicated. Recently Allen et al. [20] further confirmed that senile plaques are made up by spirochetes and reported evidence that senile plaques have characteristics of biofilms, and co-localize with Aβ.

Previous immunoelectron microscopy and immunohistochemical analyses showed that spirochetes express Aβ protein precursor (AβPP) or an AβPP-like protein, which suggests that amyloid is an integral part of spirochetes and contributes to amyloid deposition in AD [1]. Onishi et al. confirmed that *B. burgdorferi* contains amyloidogenic protein [25, 26]. Increasing number of recent reports indeed demonstrated that amyloidogenic protein is a previously overlooked integral part of the cellular envelope of many bacteria [27–31].

The goal of the present study was to investigate whether pure *B. burgdorferi* biofilms formed *in vitro* might also contain Aβ, the major component of senile plaques and whether senile plaques, similarly to pure *Borrelia* biofilms, contain DNA, an important constituent of bacterial biofilms. A panel of histochemical and immunohistochemical techniques and dark field microscopy analysis were employed to answer these questions. DNA was detected by 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) a fluorescent dye, which selectively binds DNA [32] and *in situ* hybridization was used to demonstrate spirochete-specific DNA. The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was also employed to show the presence of extracellular nuclear fragmentation in senile plaques.

The present results demonstrate that Aβ and bacterial DNA are important constituents of pure *in vitro Borrelia* biofilms and those formed in senile plaques *in vivo*. These results are additional evidence that senile plaques are formed by spirochetal colonies and correspond to bacterial biofilms. Biofilm formation in senile plaques further sustains chronic infection and inflammation and contributes to the development of slowly progressive dementia in AD.

MATERIALS AND METHODS

Cultivation of B. burgdorferi spirochetes in BSK II medium

B. burgdorferi spirochetes strains B31 cultivated from infected ticks, and strains ADB1, ADB2, and ADB3 [1, 2] cultivated from the brains of patients with neuropathologically confirmed definite AD and Lyme neuroborreliosis [2] were analyzed. All spirochetes were cultivated in BSK II medium in the following way: To 500 ml BSK medium (Sigma B 3528) containing 6% rabbit serum (Sigma R-7136) and 7% gelatin (Difco 0143-15-1), 6 mg acetyl muramic acid (Sigma A 3007) and 0.2 g N-acetyl glucosamine (Sigma A8625), Rimactan (Novartis, 420 μl) and Fosfocin (Boehringer Mannheim, 300 μl) were added. The spirochetes were cultivated at 32°C. The pH of BSK II medium was adjusted to pH 7.

In order to enhance biofilm formation, a set of 5 ml of cultivated *B. burgdorferi* spirochetes (5×10^5/ml) were exposed to various harmful conditions such as osmotic, heat shock or to strong acidic and basic conditions as previously described in detail [15]. Spirochetes cultivated at 32°C at pH 7 for the same periods of time were used as controls. Series of 50 μl samples were used to prepare smears for histochemical and immunohistochemical investigations. Unstained and stained preparations were examined by dark field or by light microscopy.

Autopsy brains of AD and control cases used in the study

Brains of 10 clinically and neuropathologically confirmed AD cases were analyzed. In all cases, spirochetes were demonstrated in the blood, brain, and cerebrospinal fluid (CSF) [1, 2] and were isolated from the brain in a modified Nogouchi medium. In three AD cases, spirochetes were also cultivated from the brain in a slightly modified Barbour-Stonner-Kelly II (BSK II) medium [1, 2]. Molecular characterization definitely identified these spirochetes cultivated in BSK II medium as *B. burgdorferi* sensu stricto (strains ADB1-3) and serological analysis confirmed that these AD patients had Lyme neuroborreliosis. The brains of four cases, without any AD-type cortical changes, where spirochetes were not observed in the blood, CSF, and brain and were not cultivated from the brain, were used as controls. The postmortem delay between death and autopsy in the ten AD and four control cases varied between 6 to 16 hours.

At autopsy, fresh, unfixed brain samples were taken from the hippocampus, inferior temporal, frontal (Brodmann's area 8-9), and parietal (Brodmann's area 39) cortical areas for direct analysis, or were frozen in liquid N_2 and stored at –80°C prior processing.

After removing these fresh and unfixed samples, brains were fixed in 10% formalin for 1 month. From formalin fixed brains, about 2–4 × 3 × 0.5-1 cm large blocks were taken at 12 representative levels from the cerebral hemispheres, basal ganglia, thalamus, and brainstem, for routine neuropathological investigations. For the semiquantitative assessment of AD-type cortical damage, additional blocks were taken from adjacent regions of the hippocampus, entorhinal cortex, inferior temporal cortex, frontal cortex (Brodmann's area 8-9), and parietal cortex (Brodmann's area 39). Following embedding in paraffin wax, five μm thick tissue sections were cut from all blocks and were used for the histochemical and immunohistochemical analyses.

Semiquantitative analysis of the density of senile plaques and neurofibrillary tangles was performed as previously described in detail [34]. Neuropathological assessment of the severity of cortical involvement was also made following Braak and Braak criteria [33]. For the definite neuropathological diagnosis of AD, the criteria recommended by Khachaturian [34], CERAD [35] and the National Institute on Aging (NIA) - Reagan Institute Working group were all fulfilled [36]. The 20 AD cases fulfilled criteria for the definite diagnosis of AD. The four, age matched control brains had no AD-type changes in the brain.

For the present study, unfixed frozen and paraffin wax embedded tissue sections from the frontal, temporal, and parietal cortex were systematically analyzed. The human brains analyzed were from the University Institute of Pathology, Division of Neuropathology, Lausanne, Switzerland. The study adhered to the tenets of the Helsinki Declaration.

Dark field microscopy and histochemical analyses of spirochetes

Samples of 50 μl of cultivated B31, ADB1, and ADB2 strains of *B. burgdorferi* spirochetes cultivated in optimal conditions at pH 7.0 and those exposed to harmful conditions [15] were used as wet preparation for dark field microscopy analysis. Smears of 50 μl samples on glass slides of these same strains of *B. burgdorferi* were also stained with Warthin & Starry and Bosma-Steiner silver impregnation techniques and with Thioflavin S, a sensitive fluorochrome, for the detection of amyloid in senile plaques in AD.

Histochemical and immunohistochemical analyses of brain sections

Unfixed and fixed tissue sections were stained with hematoxylin and eosin, cresyl echt violet, thioflavin S, Periodic Acid Schiff (PAS), Congo Red, as well as with the Maurer [37] and Gallyas silver techniques [38] for the visualization of AD-type changes, including senile plaques and neurofibrillary tangles. Unfixed cryostat and paraffin sections were also stained with Warthin-Starry and Bosma-Steiner silver techniques, which detect spirochetes.

For the visualization of AD-type lesions, paraffin sections were also immunostained with a monoclonal antibody to Aβ (DAKO, M 827, dil.1 : 50) and with polyclonal antibodies to tau (A0024, DakoCyto.,) and ubiquitin (Z 0458, DakoCyto).

A monoclonal antibody (Biogenesis 7263-1006 or Chemicon MAB995, dil.1 : 200) for the detection of bacterial peptidoglycan, a bacterial cell wall component of virtually all Eubacteria, including spirochetes, was also used as previously described in detail [39, 40].

Detection of *B. burgdorferi* specific antigens was also performed as described previously [2]. To demonstrate species-specific antigens unfixed brain

sections were post-fixed in acetone and incubated in 0.1% amylase for 5 min at 37°C. Monoclonal anti-OspA (H5332, H3T5, Symbicom, 1:50) and anti-flagellin (G 9724, H605, Symbicom, 1:50) antibodies and polyclonal antibodies B65302 R (Biodesign, 1:100) and BB-1017 (1:500) [3] were used. The specificity of these mono- and polyclonal antibodies was previously tested by western blot analysis [2].

For immunostaining, the avidin-biotin-peroxidase technique was used. Following 24, 48, or 72 h incubation with a primary antibody at 4°C, the sections were incubated with the appropriate secondary antibody. For monoclonal antibodies, a biotinylated F(ab) fragment of affinity isolated rabbit anti-mouse immunoglobulin (Dako, E413) was used. The immunoreaction was revealed by diaminobenzidine (DAB) alone, or with nickel-ammonium sulfate as previously described [41]. Immunostaining was also performed with various anti-*B. burgdorferi* antibodies using FITC tagged anti-mouse or anti-rabbit secondary antibody. The green fluorescence of the positive immunoreaction was analyzed with a Zeiss fluorescent microscope. Brain tissue sections of control cases without brain lesions were immune-stained in the same way. Frozen sections immunostained in the absence of the primary antibody or with an irrelevant mono- or polyclonal antibody were used as controls.

DNA labeling

The fluorochrome 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI), was used to detect DNA. From the 10 neuropathologically confirmed AD cases, and four control cases, 7 μm thick cortical sections were cut on a cryostat, postfixed with methanol for 2 min and stained with 3 μg/ml of DAPI (Boehringer, 236 276) in methanol for 15 min at 37C. The sections were rinsed in distilled water for 5 min and were mounted with gum arabic, coversliped and examined with a fluorescence microscope either in UV light, using G 365/11 excitation and LP 397 barrier filters, or using Bp 485/20 excitation and LP 520 barrier filters. Frozen sections of control cases where also stained with DAPI. In order to remove DNA, another set of sections before staining with DAPI was treated with 1 mg/ml of DNase I (Boehringer, 1284 932) diluted in PBS containing 5mmol/ml of Mg++, at pH 7.8 at 37°C for 3 h. The same procedure was also carried out using RNase free DNase I (Boehringer, 776 785). In order to eliminate the possibility of an unspecific binding of the DAPI to amyloid, a set of DNase I treated sections were post-stained with thioflavin S, widely used for the demonstration of amyloid in AD [32]. Smears of strains B31, ADB1 and ADB2 of *B. burgdorferi* were treated and examined in the same manner.

Detection of spirochete-specific DNA by in situ hybridization

In the three AD cases, where *B. burgdorferi* was cultivated from the brain, *B. burgdorferi* specific bacterial DNA was also detected using *in situ* hybridization. Hybaid, OmniGene thermal cycler was used, which was equipped with a Satellite Module of *In-Situ* block. Paraffin sections (5 μm) and frozen sections (10 or 20 μm) were both employed as previously described [2]. The paraffin sections were de-waxed in xylene, hydrated in 99%, and 95% ethylene and rinsed in pure water 2×3 min. On both frozen and paraffin sections, endogenous peroxidase was blocked by treatment in methanol containing 3% H_2O_2. The sections were treated with 1% hot SDS (70°C) for 5 min, with Lysozyme (25 000 U/ml in PBS, pH 5.5 at 37°C) for 5 min and with Proteinase K (10 μg/ml in 50 mM Tris-HCL, pH 7.6 at 37°C) for 30 min. Following each treatment, the sections were washed in pure water 3×10 min. The sections were post-fixed for 20 min with 1% paraformaldehyde in PBS containing 50 mM $MgCl_2$, rinsed with three changes of pure water, and dried in a series of ethanol washes. The sections were incubated with a pre-hybridization solution (1 μl 0.5M Tris HCl, pH 7.4, 50 μl 20-X- SSC, 1 μl 0.05 M EDTA, 100 μl of 50% dextran sulfate, 250 μl formamide, and 98 μl of pure water for a total volume of 500 μl) in the humidity chamber of the thermal cycler at 42°C for 1 h. The pre-hybridization solution was then replaced by the hybridization solution containing 100 ng of probe labeled by nick-translation with Digoxigenin (OspA gene BBB012, SN3, position 360–426); flagellin gene BBB032, WK3, position 396–425 purchased from GENSET). The nucleotide sequence of the probes was: 5'–CAA TGG ATC TGG AGT ACT TGA AGG GGT AAA AGC T–3' and 5'-AAT GCA CAT GTT ATC AAA CAA ATC TGC TTC–3', respectively. The sections were coversliped, and 10 min incubation at 100°C was followed by an overnight incubation at 42°C in the humidity chamber of Hybaid cycler. Post-hybridization washes were in an equal mixture of formamide and 2-X-SSC, pH 7 at 42°C for 2×20 min and in 0.1-X-SSC, 2 mg $MgCl_2$, 0.1% Triton-X-100 at 60°C for 30 min. After a rinse in TBS 3×5 min, the sections were treated

with a blocking solution containing normal rabbit serum diluted 1 : 5, 3% bovine serum albumin and 0.1% Triton-X-100 in TBS for 1 h. For the detection of hybridization products anti-digoxigenin alkaline phosphatase or peroxidase conjugates were used. The alkaline phosphatase substrate solution or DAB were used as chromogens for the visualization of reaction products. Control sections without specific probes and sections of control patients without AD lesions were used as negative controls.

TUNEL Assay

To analyze whether *in situ* DNA fragmentation occurs in senile plaques, the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was performed, according to the manufacturer's instructions. Paraffin and fresh cryostat sections from the frontal cortex of three AD cases and two controls were post-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by two washes in PBS. The paraffin sections were heated in 80 ml TRIS buffer in a microwave oven at 800 W for 5 min. On both paraffin and frozen sections the endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Following 3×5 min wash with PBS, they were treated with Proteinase K (20 μg/ml) at room temperature for 15 min. Following another rinse with PBS (3×5 min) the sections were fixed with 4% paraformaldehyde for 5 min and then treated with 1% Triton in 0.1% sodium citrate on ice for 2 min. Slides were washed again with PBS and incubated with the TUNEL reaction mixture (45 μl Tunel Label: Boehringer, 1767291 and 5 μl Tunel Enzyme Boehringer, 1767305). The reaction was stopped immersing the slides in $2 \times$ SSC at room temperature. The nucleotide mixture contained fluorescein-iso-thio-cyanate (FITC) labeled dUTP. In order to convert the fluorescence into a visible signal with light microscopy, the sections were treated with horseradish peroxidase (HRP)-labeled anti-FITC antibody for 30 min at 37°C (Boehringer, 1426320) and then were washed three times in PBS. The enzymatic reaction was revealed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 10 min at room temperature. The sections were counterstained with hematoxylin. Sections treated the same way but with omission of TdT enzyme from the reaction mixture were used as controls. DNA fragmentation was examined directly after the TUNEL reaction using fluorescence microscopy.

RESULTS

B. burgdorferi strain B31 cultivated from infected tick and strains ADB1-3 cultivated from the brains of AD cases all form biofilm *in vitro* and show identical morphology and biochemical properties. All strains form colony-like aggregates or biofilms enclosing numerous atypical and granular spirochetal forms. Spirochetes with the usual spiral or vegetative form are frequently seen at the periphery of these pure spirochetal biofilms. The number of biofilms was higher in 4–6 week-old cultures compared to 1-week-old cultures. Similarly, the number of *B. burgdorferi* biofilms was higher in various harmful conditions compared to those cultivated in optimal condition. Figure 1 illustrates the morphology of these pure spirochetal biofilms and some of their characteristics. They can be visualized with dark field microscopy as illustrated for strains B31 (Fig. 1A) and ADB1 (Fig. 1B), respectively. These pure *B. burgdorferi* biofilms, similarly to senile plaques, are argyrophilic when stained with silver impregnation techniques (Fig. 1C). They also contain species-specific antigens when immunostained with various anti-*B. burgdorferi* antibodies. *B. burgdorferi* biofilm expressing outer surface protein A (OspA) is illustrated in Fig. 1C. Pure *B. burgdorferi* biofilms of all strains exhibited green thioflavin S fluorescence (Fig. 1E), similar to that of senile plaques (Fig. 1F). When these pure *B. burgdorferi* biofilms were immunostained with anti-AβPP (Fig. 1G) and anti-Aβ antibodies, which are routinely used for the detection of Aβ in senile plaques (Fig. 1H), they exhibit positive immunoreaction to both AβPP and Aβ.

DNA labeling with DAPI of smears of pure *B. burgdorferi* biofilms and brain cortical sections of the 10 AD cases analyzed, in UV light, showed silver-white fluorescence of spirochetal biofilms and senile plaques. Green DNA fluorescence of pure *B. burgdorferi* biofilms (Fig. 2A) and senile plaques (Fig. 2B, C) are visible when Bp 485/20 excitation and LP 520 barrier filters are employed. On brain sections, in addition to fluorescent brain cell nuclei, senile plaques exhibit fluorescence of DNA as illustrated in a familial AD case (Fig. 2B) and in the AD case where *B. burgdorferi* strain ADB1 was cultivated from the brain (Fig. 2C). In the four control cases, without AD-type changes, only nuclei of brain resident cells showed fluorescence (Fig. 2D). DNase I or RNase free DNase I pretreatment abolished DNA fluorescence of both brain cell nuclei and senile plaques (Fig. 2E). When DNase treated brain

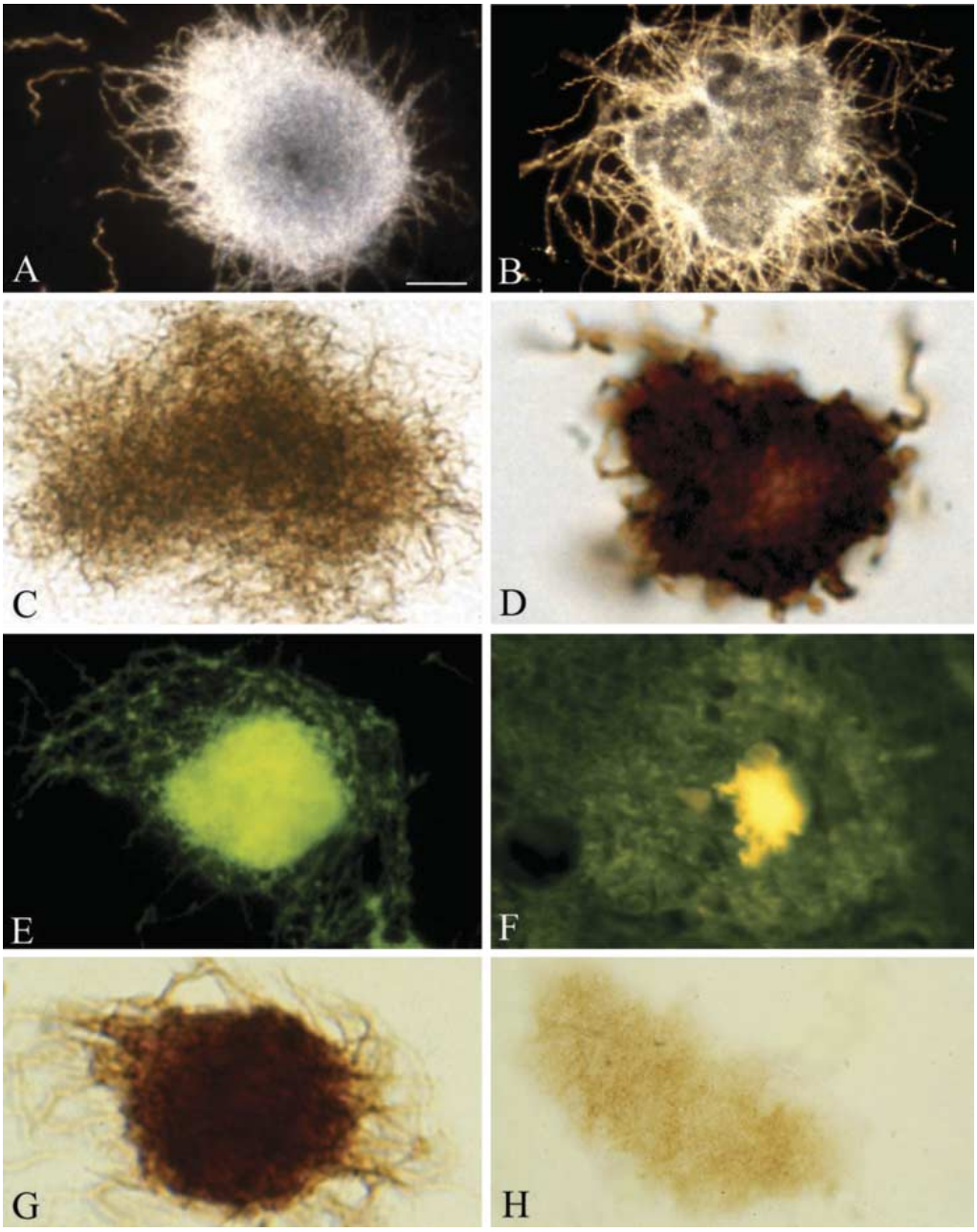

Fig. 1. Pure *in vitro B. burgdorferi* biofilms contain Aβ an important component of senile plaques. A, B) Dark field microscopy images of pure *B. burgdorferi* biofilms of reference strain B31 cultivated from infected tick (A) and strain ADB1 cultivated from the brain of an AD patient with confirmed chronic Lyme neuroborreliosis (B). C) Pure *B. burgdorferi* biofilm of strain ADB2 stained with Warthin and Starry silver technique for the detection of spirochetes. D) Pure *B. burgdorferi* biofilm of strain ADB2 immunostained with anti-OspA monoclonal antibody exhibiting positive immunoreaction. E) Green thioflavin S fluorescence of *in vitro* formed *B. burgdorferi* biofilm of strain ADB2. F) Thioflavin S fluorescence of a senile plaque in the frontal cortex of an AD patient where *B. burgdorferi* ADB2 strain was cultivated from the brain. G) *In vitro* formed *B. burgdorferi* biofilm (strain B31) immunoexpressing AβPP; H) *In vitro B. burgdorferi* biofilm of strain ADB1 exhibiting positive Aβ immunoreaction. Scale bar = A: 15 μm for A-C and E-H and 10 μm for D.

sections were post-stained with thioflavin S, senile plaques showed strong yellow-green thioflavin S fluorescence indicating that DNase I does not abolish amyloid staining (Fig. 1F). As bacteria are lacking nuclear membrane and their DNA is diffusely distributed in their cytoplasm, DNA labeling with DAPI

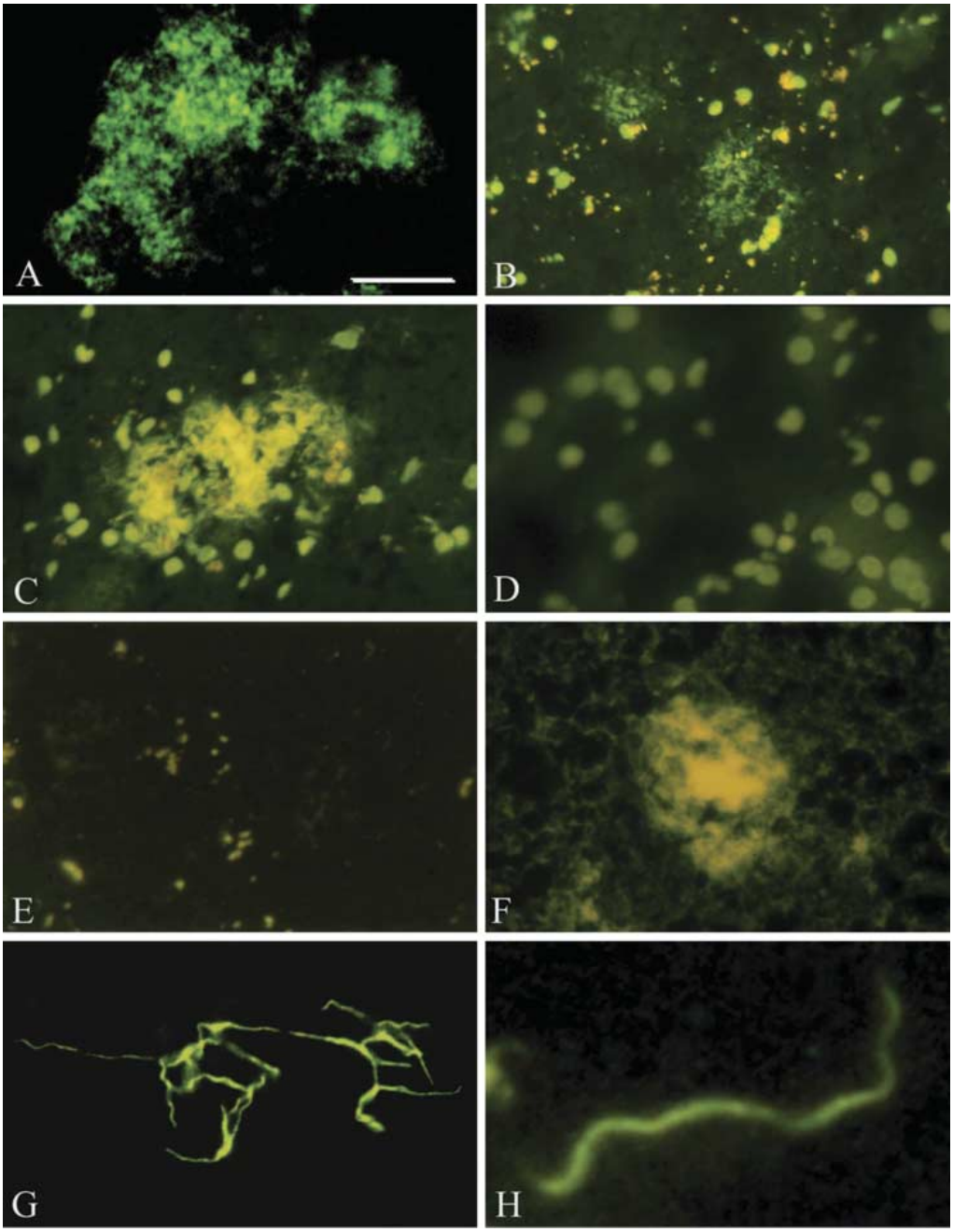

Fig. 2. Pure *in vitro B. burgdorferi* biofilm and senile plaques both contain DNA, an important constituent of biofilms. A) Smear of *in vitro B. burgdorferi* biofilm of ADB2 strain stained with DAPI and exhibiting green fluorescence, when examined with Bp 485/20 excitation and LP 397 barrier filters. Similar DAPI fluorescence of senile plaques is seen in frontal sections of a familial (B) and an AD case where *B. burgdorferi* spirochetes were cultivated from the brain (C). D) On DAPI-stained frontal section of a control case only brain cell nuclei are visible. E) Following DNase treatment of a frontal cortical section of an AD case, the DAPI fluorescence of resident cell nuclei and senile plaques both disappeared. F) DNase treated AD cortical section stained with Thioflavin S. Senile plaque exhibits a yellow-green fluorescence indicating that DNase pretreatment does not abolish amyloid staining of the plaques. G, H) DAPI fluorescence of *B. burgdorferi* spirochetes, revealing their typical helical structure. Photomicrographs E and F were reproduced from [32] with kind permission of the editor of *Journal of Spirochetal and Tick-borne Diseases*. Scale bar = A: 60 μm, B: 200 μm, C: 120 μm, D: 100 μm, E: 120 μm, F: 10 μm; H: 2 μm.

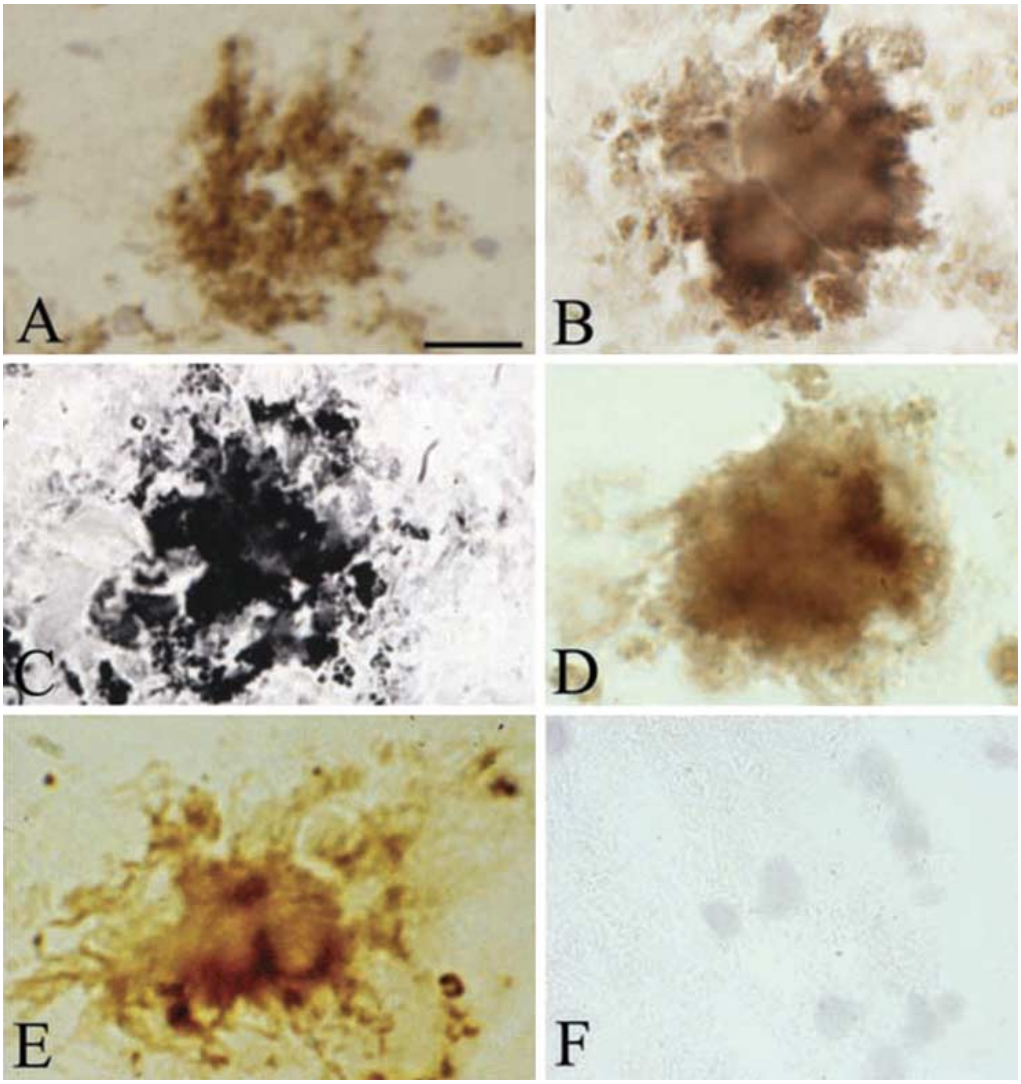

Fig. 3. Senile plaques contain spirochete-specific DNA. Photomicrographs of spirochetal colonies or biofilms in an AD case with confirmed Lyme neuroborreliosis where *B. burgdorferi* spirochetes (ADB1) were cultivated from the brain. A) Positive Aβ immunoreaction of senile plaque. B) Senile plaque of the same AD case, as in A, exhibiting strong immunoreaction for bacterial peptidoglycan. C, D) Photomicrographs showing *B. burgdorferi* antigens in senile plaques immunostained with a polyclonal anti-*B. burgdorferi* antibody (C) and with a monoclonal anti-OspA antibody (D). E) *B. burgdorferi* specific DNA detected by *in situ* hybridization in senile plaque of an AD patient where ADB1 strain was cultivated. F) Cortical section of a control case immunostained with a monoclonal anti-OspA antibody showing no immunoreaction. Scale bar = A-E: 40 μm, F = 25 μm. Photomicrograph E is a reproduction of Fig. 2b of [7].

reveals their characteristic morphology. DNA florescence of a small group (Fig. 2G) and a single spirochete (Fig. 2H) when stained with DAPI demonstrates, by the diffusely located bacterial DNA, the helical shape of *B. burgdorferi*.

Immunodetection of Aβ in senile plaques is routinely used for the neuropathological diagnosis of AD. When cortical sections of the 10 AD cases analyzed, including in the three AD cases where *B. burgdorferi* were cultivated from the brain senile plaques showed strong Aβ immunoreaction with all of the anti-Aβ antibodies used (Fig. 3A). Senile plaques are also immonoreactive to the highly specific anti-bacterial peptidoglycan antibody (Fig. 3B). In the three AD cases where *B. burgdorferi* was also cultivated from the brain in BSK II medium (strains ADB1-3), not only Aβ and bacterial peptidoglycan but *B. burgdorferi* specific antigens are also present in senile plaques. Figure 3C and D illustrate the presence of *B. burgdorferi* specific antigens in senile

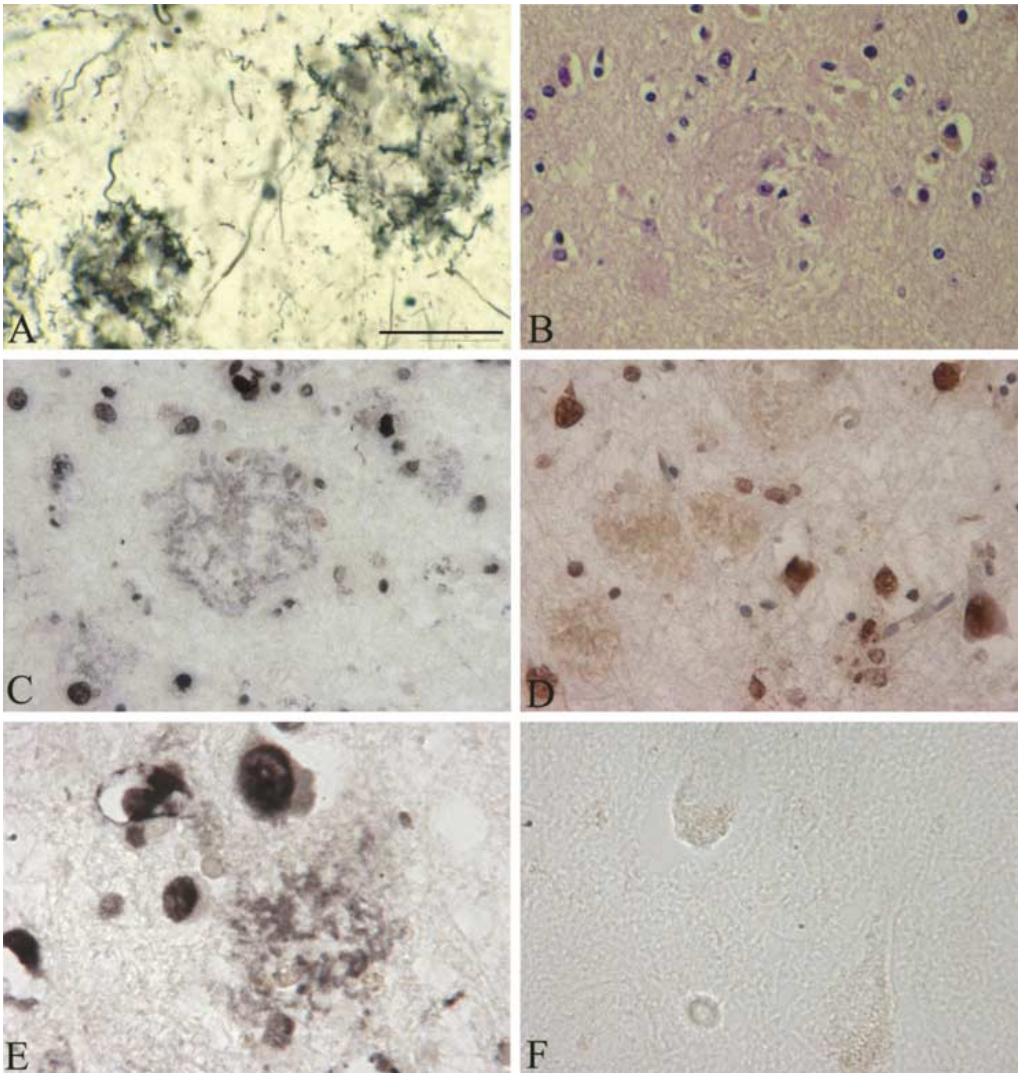

Fig. 4. I*n situ* DNA fragmentation in senile plaques using the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay. A) Senile plaques in an AD case where *B. burgdorferi* (strain ADB1) was cultivated from the brain. Frozen section stained with Maurer technique used for the detection of senile plaques in AD. B) Paraffin section stained with Haematoxylin and eosin, in the same case as in A, showing senile plaques in the frontal cortex. C-E) Frozen (C and E) and paraffin (D) sections from the frontal associative area (Brodmann's area 8-9) of the AD case, where *B. burgdorferi* strain ADB2 was cultivated from the brain. The TUNEL assay demonstrates i*n situ* DNA fragmentation in black (C and E) or in brown (D) color. The majority of cells showing apoptosis are large nuclei of neurons, but some glial cells also exhibit TUNEL positive nuclei. Scale bar = A-C: 150 μm, D: 200 μm, E, F: 80 μm.

plaques as revealed by polyclonal and monoclonal anti-*B. burgdorferi* antibodies, respectively. In these three AD cases where the ADB strains were cultivated from the brain, *B. burgdorferi* specific DNA is demonstrated in senile plaques by *in situ* hybridization (Fig. 3E). The extranuclear localization of *B. burgdorferi* specific DNA excluded the possibility of an unspecific DNA labeling of host cell nuclei. Control sections where specific *B. burgdorferi* antibodies or probes were omitted were negative (Fig. 3F). Cortical sections of control cases without AD-type changes and where spirochetes were not cultivated from the brain did not show Aβ and bacterial peptidoglycan immunoreaction or *B. burgdorferi* specific antigens or DNA.

In the three AD cases analyzed, the TUNEL assay shows DNA fragmentation not only of various brain cell nuclei but in a number of senile plaques as well, in an extracellular location. Figure 4 illustrates DNA fragmentation in senile plaques as revealed by the TUNEL assay in the frontal cortex of an AD case where *B. burgdorferi* was cultivated from the brain.

Silver impregnation technique [37] (Fig. 4A) and hematoxylin and eosin stain (Fig. 4B) show the presence of typical senile plaques in the frontal cortex in this AD case. DNA fragmentation in a number of brain cell nuclei, mostly in neurons, and in some glial and endothelial cells is visible. *In situ* DNA fragmentation as revealed by TUNEL in a subset of senile plaques located extracellularly with respect to resident brain cells is also apparent on both frozen (Fig. 4C, E) and paraffin (Fig. 4D) sections. The extracellular distribution of this DNA fragmentation, located mostly in filamentous structures is similar to the DNA detected by DAPI and to spirochete-specific DNA revealed by *in situ* hybridization. Only few neurons exhibited nuclear fragmentation and extracellular TUNEL reaction was not present in the brains of the two control cases analyzed.

DISCUSSION

Amyloidogenesis is the aggregation of soluble proteins into detergent-insoluble filamentous structures, which have distinct biochemical and biophysical properties, including resistance to proteinase K treatment, beta-sheet structure and affinity for binding thioflavin S and Congo red.

Recent observations indicate that aggregated masses or colonies of *B. burgdorferi* spirochetes formed *in vitro* have characteristics of biofilms [21]. Even more recently, Allen et al. [20] demonstrated that senile plaques, which were shown to correspond to spirochetal masses or colonies, have properties of biofilm. *B. burgdorferi* spirochetes also form such colonies following infection of mammalian cells or organotypic cultures *in vitro* [16], which exhibit Aβ-immunoreaction and are undistinguishable from senile plaques in AD.

We anticipated that if pure *B. burgdorferi* biofilms formed *in vitro* comprise AβPP and Aβ would indicate, as previously suggested [1], that bacterial amyloid is an important component of senile plaques in AD. This would also indicate that bacterial amyloid is an important constituent of biofilms, and contribute to the formation of the slimy material covering and protecting bacteria in biofilms. Accordingly, we analyzed and compared the amyloid characteristics of *in vitro* formed pure spirochetal biofilms with those formed in senile plaques *in vivo*. Ten definite AD cases where spirochetes were cultivated in a modified Nogouchi medium and four control cases without AD-type changes were analyzed. In three of the 10 AD cases with clinically and neuropathologically confirmed Lyme neuroborreliosis, spirochetes were also cultivated in BSK II medium and using molecular techniques were definitely identified as *B. burgdorferi* sensu stricto (strains ADB1, ADB2, and ADB3) [2]. The analysis of the characteristics of these ADB *Borrelia* strains allowed us to directly compare the characteristics of pure *in vitro Borrelia* biofilms with those formed *in vivo* in senile plaques.

All spirochetal strains analyzed (B31 and ADB1-3) have the ability to form biofilms *in vitro.* Biofilms formed by these various strains have similar morphological and chemical properties. As observed by dark field microscopy and by the presence of species-specific antigens, the formation of spirochetal biofilms *in vitro* was enhanced in older cultures with a higher spirochetal cell density and in various harmful conditions, compared to those cultivated in optimal conditions.

Thioflavin S, which binds amyloid with high affinity, strongly binds pure *B. burgdorferi* biofilms, indicating that they contain amyloid. As AβPP and Aβ are important components of senile plaques, smears of pure *Borrelia* biofilms of all strains were immunostained with a set of mono- and polyclonal antibodies specifically recognizing AβPP and Aβ. Pure biofilms of all *Borrelia* strains studied exhibited a positive immunoreaction, demonstrating that similarly to senile plaques pure *Borrelia* biofilms contain AβPP and Aβ. These results further confirm previous observations based on immunohistochemical and immunoelectronmicroscopical analyses that AβPP or an AβPP-like amyloidogenic protein is an integral part of spirochetes. This indicates that bacterial amyloid contributes to Aβ deposition in AD [1]. That amyloid is an integral part of spirochetes, namely of *B. burgdorferi*, was further confirmed by Ohnishi et al., who reported that the BH (9–10) peptide on a beta-hairpin segment of *B. burgdorferi* OspA, forms amyloid fibrils *in vitro* that is similar to human amyloidosis [25, 26]. These results are in agreement with previous observations that *B. burgdorferi* spirochetes form Aβ immunoreactive colonies, similar to senile plaques *in vitro* following infection of primary neuronal and glial cells or organotypic cultures [16]. *Borrelia* colonies or biofilms formed *in vitro* adhering to cells or free floating in the medium, both exhibited thioflavin S fluorescence and immunoexpressed Aβ [16].

These results are also in agreement with the observations that the cortical spirochetal colonies of *T. pallidum* in syphilitic dementia are Aβ immunoreactive [5, 42].

Amyloid proteins constitute a previously overlooked integral part of the cellular envelope of many bacteria [27, 29–31]. Bacterial amyloids are biologically functional molecules, which play an important role in virulence, invasion, and host cell destruction [27–30]. Bacterial amyloids are involved in bacterial cell-cell interactions, in their attachment to inert solid surfaces, and in spore and biofilm formation [28].

All these observations indicate that bacterial amyloid is present in senile plaques.

Host cells and bacteria, during host-pathogen interactions, use similar molecular mechanisms to induce host cell lysis and bacteriolysis. Recent observations reveal that Aβ, the most important biological marker of AD, is an innate immune molecule, and shares properties with antimicrobial peptides [43]. Soluble $A\beta_{1-42}$ oligomers form channels on lipid cell membranes and cause Ca^{2+} influx and cell destruction [44]. Channel formation in the membrane of targeted host cells triggering cellular ion imbalance is also a form of bacterial attack [45, 46]. This is also in harmony with the present findings that bacterial Aβ with neurotoxic activities and host derived Aβ with antimicrobial properties both are constituents of amyloid deposits in AD. Further studies will be required to distinguish between host and bacteria derived amyloid and determine whether host cell destruction predominates over bacteriolysis in chronic sustained infections and determine whether the lower level of inflammation in chronic disorders might be insufficient to clear invading pathogens.

The present observations also demonstrate that bacterial amyloids are critical components of biofilms and play an important role in biofilm formation, and in formation of the slimy cover, which confers to bacteria protection against harmful conditions and host immune reactions.

DAPI is a fluorescent dye, which binds selectively to DNA [47] and forms strongly fluorescent DNA-DAPI complexes with high specificity, yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence [47, 48]. Its specificity was found to be similar to that of the fluorescent DNA-binding benzimidole derivative Hoechst 33258 [47, 48]. DAPI, by its specificity and sensitivity, is frequently used for the detection of Mycoplasma infection in cell cultures [47, 49]. Mitochondrial DNA binds DAPI, but at levels imperceptible by routine fluorescent microscopy [49]. Bacteria being prokaryotic cells contain DNA, but they differ from eukaryotic cells in that the nuclear material is not surrounded by a limiting nuclear membrane. We therefore expected that DAPI binds the DNA of spirochetes as well and show their characteristic helical shape [32]. As demonstrated here, reference spirochetes by their DNA content can be visualized by DAPI [32]. If senile plaques are indeed formed by spirochetes and correspond to biofilms, they would consequently contain DNA outside resident cell nuclei and exhibit DAPI fluorescence. Here we demonstrate that DAPI indeed binds to senile plaques indicating that they contain DNA. The extracellular distribution of DNA in filamentous structures, similar to those seen in individual spirochetes, together with historic and recent observations showing that senile plaques are made up by spirochetes and correspond to biofilm indicate that the extracellular DNA in senile plaques detected by DAPI corresponds to bacterial DNA. DNA-se I treatment abolishes not only the DAPI fluorescence of host cell nuclei but that of senile plaques as well. The fact that DNase pretreatment did not abolish thioflavin S fluorescence of senile plaques [32] indicates, in agreement with Russel et al. [47], the specificity of DAPI as a sensitive DNA fluorescent stain of eukaryotic and prokaryotic DNA. The present observations further indicate that spirochetes are causal agents in AD and in an analogous way to *T. pallidum* various periodontal pathogen spirochetes, *B. burgdorferi*, and other, still uncharacterized virulent spirochetes can cause dementia, cortical atrophy, and amyloid deposition in AD.

In order to confirm the spirochetal origin of DNA detected in senile plaques by DAPI, specific nucleic acid probes detecting *B. burgdorferi* specific DNA were also used in AD cases with clinically, serologically, and neuropathologically confirmed Lyme neuroborreliosis where *B. burgdorferi* was cultivated from the brains. In the brains of these AD cases, *B. burgdorferi*-specific spirochetal DNA was demonstrated in senile plaques using *in situ* hybridization, indicating that the DNA detected by DAPI is indeed spirochetal DNA.

The most common mode of programmed cell death is apoptosis. Many of the morphological and biochemical responses associated with apoptosis in eukaryotes also occur in prokaryotes. One of these markers, which occurs in both, is DNA fragmentation [50–52]. DNA fragmentation of apoptotic bacterial cells similarly to those of apoptotic host cell nuclei can be demonstrated by TUNEL assay, which incorporates fluorescein-dUTP into the ends of fragmented DNA [52]. If senile plaques are indeed spirochetal biofilms, we expected that fragmentation of bacterial DNA might also be present in senile

plaques. Using TUNEL, extracellular DNA fragmentation is present in a subset of senile plaques. The location and distribution of extracellular DNA fragmentation is identical to those of DAPI fluorescence and spirochete-specific DNA as revealed by *in situ* hybridization. This indicates that this extracellular DNA fragmentation in senile plaques revealed by TUNEL corresponds to bacterial apoptosis in spirochetal colonies or biofilms formed in senile plaques.

A number of resident brain cells, predominantly neurons, and to a lesser extent glial and endothelial cells also showed DNA fragmentation by TUNEL. DNA fragmentation in astrocytes is rarely seen. These results indicate that spirochetes cause functional damage and cell death in host tissues. This is in agreement with previous observations that spirochetes induce apoptosis of resident brain cells, mostly of neurons *in vivo* [53, 54]. Apoptosis of neurons and glial cells was also observed following *B. burgdorferi* infection of primary mammalian cells and organotypic cultures *in vitro* [15].

During infection, pathogens employ a broad range of strategies to overcome antigenic recognition, phagocytosis, and complement lysis. Blockade of the complement cascade allows their survival even in immune competent hosts. If pathogens are not recognized by the host immune systems or in the absence of cell-mediated immune responses, the microorganism can spread freely and accumulate in the affected host tissues. Under such conditions, the microorganisms establish chronic infection, inflammation, and progressive tissue damage. Biofilms protect invading spirochetes from destruction by the host immune reactions and in the maintenance of chronic infection. These spirochetal agglomerations or biofilms in senile plaques in accordance with Steiner are a form of resistance to adverse conditions, and a source for newly growing spirochetes under more favorable conditions [12].

That extracellular DNA, an important component of biofilms, is also present in senile plaques indicates that senile plaques are indeed made up by spirochetes, and contain bacterial amyloid in the form of Aβ, and confirm recent observations [20] that senile plaques indeed correspond to biofilms. The present results further highlight that amyloid is an essential component of *in vitro* and *in vivo* formed biofilms. Microbial amyloids, through interaction with host proteases, contribute to bacterial virulence, to colonization of the host and invasion of host cells.

Evasion of spirochetes from host immune reactions initiates and sustains the proliferation of spirochetes and their aggregation leading to biofilm formation in the form of senile plaques in AD. These results explain why dementia appears years or decades following the primary spirochetal infection and why long standing chronic bacterial infections are frequently association with amyloid deposits. They also indicate that in various chronic inflammatory disorders, which are associated with amyloidosis the involvement of bacteria should be investigated.

Reports of an association between infection and AD are not confined to spirochetes. *Chlamydia pneumonia. Porphyromonas gingivalis, Proprionobacterium acne, Helicobacter pylori* and other bacteria were also found to be associated with AD [55–61] and mice exposed to *Chlamydia pneumoniae* developed AD-like amyloid plaques in the brain [62]. Herpes virus type 1 and other viruses were also demonstrated in the brain in AD [64, 65]. As spirochetes frequently co-infect with other bacteria and various viruses to consider that senile plaques may correspond to multimicrobial biofilms is important.

CONCLUSION

Biofilm formation confers to bacterial resistance to antibiotics and other anti-microbial agents and contributes to the establishment of chronic infection. Recently it was demonstrated that spirochetes are able to form biofilms *in vitro* and senile plaques have characteristics of biofilm.

If senile plaques indeed correspond to biofilms, bacterial amyloid and DNA should be the component of both pure spirochetal biofilms formed *in vitro* and biofilms in senile plaques formed *in vivo*. Ten definite AD cases and four controls without AD-type changes were analyzed. Three AD cases with clinically and neuropathologically confirmed Lyme neuroborreliosis, where *B. burgdorferi* spirochetes were cultivated from the brain and were definitely identified as *B. burgdorferi* sensu stricto, enabled us to compare pure *B. burgdorferi* biofilms formed *in vitro* with *B. burgdorferi* biofilms formed in senile plaques in AD *in vivo*.

The present results reveal that AβPP and Aβ are not only important components of senile plaques but of pure *in vitro* formed *B. burgdorferi* biofilms as well, indicating that bacterial amyloid together with host derived Aβ are important constituents of senile plaques and support previous observations that AβPP or an AβPP-like amyloidogenic protein is an integral part of spirochetes and contribute to amyloid

deposition in AD [1]. The results also indicate that the known physical and chemical properties of amyloid strongly contribute to the protective effect of biofilms against harmful conditions and against host immune responses.

Bacterial DNA (as visualized by DAPI) and spirochete-specific DNA (as detected by *in situ* hybridization) are further indication that senile plaques are made up by spirochetes and correspond to biofilms. Bacterial apoptosis is also present in a subset of senile plaques and is indicative of the bacterial or biofilm nature of senile plaque and represents chemical evidence of spirochetal cell death. Nuclear fragmentation of resident cell nuclei indicates that spirochetes, including *B. burgdorferi* cause DNA fragmentation of neuronal, glial, and endothelial cells in AD.

The present findings strengthen previous observations that AβPP or an AβPP-like amyloidogenic protein is an integral part of spirochetes, and contribute to Aβ deposition in AD.

These observations also highlight the direct role of *B. burgdorferi* and other spirochetes in the chronic manifestations of neurospirochetoses, and indicate that spirochetes play a causal role in AD and in Lyme dementia.

Spirochetal biofilms in senile plaques strongly contribute to the long latent stage and to persisting infection in chronic neurospirochetoses, including in Lyme disease. The ability of spirochetes to evade host defenses, the formation of bacterial biofilms in senile plaques, together with the ability of spirochetes to locate intracellularly and form more resistant atypical forms all contribute to establish and sustain chronic infection and inflammation and lead to progressive dementia, sometimes decades following the primary infection.

As targeted therapies are available, it is imperative to stop and prevent, as early as possible, the devastating consequences of various chronic spirochetoses, including Lyme disease and periodontal disorders.

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The author's disclosure is available online (http://j-alz.com/manuscript-disclosures/16-0451r1).

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Determining the Presence of Periodontopathic Virulence Factors in Short-Term Postmortem Alzheimer's Disease Brain Tissue

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Abstract. The aim of this study was to establish a link between periodontal disease and Alzheimer's disease (AD) with a view to identifying the major periodontal disease bacteria (*Treponema denticola*, *Tannerella forsythia*, and *Porphyromonas gingivalis*) and/or bacterial components in brain tissue from 12 h postmortem delay. Our request matched 10 AD cases for tissue from Brains for Dementia Research alongside 10 non-AD age-related controls with similar or greater postmortem interval. We exposed SVGp12, an astrocyte cell line, to culture supernatant containing lipopolysaccharide (LPS) from the putative periodontal bacteria *P. gingivalis*. The challenged SVGp12 cells and cryosections from AD and control brains were immunolabeled and immunoblotted using a battery of antibodies including the anti-*P. gingivalis*-specific monoclonal antibody. Immunofluorescence labeling demonstrated the SVGp12 cell line was able to adsorb LPS from culture supernatant on its surface membrane; similar labeling was observed in four out of 10 AD cases. Immunoblotting demonstrated bands corresponding to LPS from *P. gingivalis* in the SVGp12 cell lysate and in the same four AD brain specimens which were positive when screened by immunofluorescence. All controls remained negative throughout while the same four cases were consistently positive for *P. gingivalis* LPS ($p = 0.029$). This study confirms that LPS from periodontal bacteria can access the AD brain during life as labeling in the corresponding controls, with equivalent/longer postmortem interval, was absent. Demonstration of a known chronic oral-pathogen-related virulence factor reaching the human brains suggests an inflammatory role in the existing AD pathology.

Keywords: Alzheimer's disease, lipopolysaccharide, periodontal disease, *Porphyromonas gingivalis,* postmortem

INTRODUCTION

Periodontal disease (PD) is a chronic immuno-inflammatory disease initiated by complex polymicrobial subgingival biofilm. This results in the inflammatory destruction of tooth supporting tissues, including the gingivae, periodontal ligament, and alveolar bone [1]. Analysis of the human oral microbiota has revealed more than 700 bacterial species in the oral cavity and over 400 species in the subgingival plaque of healthy and PD oral biofilms [2]. These pathogens interact with the host and result in significant systemic inflammation characterized by the induction of proinflammatory cytokines, chemokines, and exaggerated host immune responses [3, 4].

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Periodontal pathogens adhere to and colonize the subgingival pocket in the form of a biofilm and the net effect of this bacterial biofilm community is to maintain a persistent chronic infection within the host. Several studies suggest that PD-associated bacteria can penetrate gingival tissues and enter the bloodstream during chewing, tooth brushing, or dental procedures and may induce a recurrent transient bacteremia [5, 6]. Thus, periodontal lesions are recognized as continually renewing reservoirs for the systemic spread of bacteria, antigens, and cytokines along with other proinflammatory mediators. Once the bacteria, virulence factors, and/or indirectly released inflammatory mediators reach remote body organs, it has been postulated that they may induce similar inflammatory responses, resulting in the tissue-specific pathology. Chronic PD has been linked to several systemic diseases such as atherosclerotic vascular disease [7], adverse pregnancy outcome [8, 9], diabetes [10, 11], respiratory diseases [12], renal disease [13], rheumatoid arthritis [14, 15], and Alzheimer's disease (AD) [16].

Thus, a link between periodontitis and AD has been proposed [16] although the strength and relevance of the association remains to be fully investigated. Besides oral pathogens being found in the aged human brains, viruses such as Herpes Simplex Virus Type 1 [17] and diverse bacterial infections, including *Chlamydia pneumoniae* [18] and *Borrelia burgdorferi* [19], have also been implicated in the pathogen-associated etiology of the late onset AD, as recently reviewed by Miklossy [19, 20]. The pathological characteristics of AD are the extracellular fibrillar amyloid-β (Aβ) deposits and the neurofibrillary tangles [21]. However, elderly cognitively unimpaired individuals also show these lesions in the brain but to a lesser degree than that expected to cause dementia [22].

Brain inflammation behind the blood-brain barrier (BBB) differs from inflammation in the periphery by the relative absence of leukocytes (including neutrophils, monocytes, B cells, and T cells) and antibodies; however, the presence of activated microglial cells is the key contributor of inflammation in the brain [23]. Activated microglial cells express a range of proinflammatory cytokines [23, 24] and are capable of recognizing the non-self-pathogen-associated molecular patterns (PAMPs) on bacteria and their cellular debris. However, the current view regarding the inflammatory response in the AD brain is viewed as being a downstream consequence of the Aβ accumulation resulting in the activation of microglia; this initiates a pro-inflammatory cascade and brings about the local release of potentially neurotoxic substances such as cytokines, complement factors, and reactive oxygen species [24]. Interestingly, experimentally induced microbial infections and/or their virulence factors also appear to contribute to CNS inflammation and in some cases to lead to Aβ deposition [25–28].

Inflammation also plays a key part in the oral cavity; the immediate response to periodontal pathogens and their endotoxins is to activate the local and systemic innate immune responses [29] leading to the recruitment of inflammatory cells (macrophages, T and B cells) that secrete cytokines [(interleukin (IL)-1, IL-6, tumour necrosis factor-alpha (TNF-α), and interferon-gamma (INF-γ) [29–31]. The inability of the innate immune system to remove pathogens such as *P. gingivalis* [32–35] results in progressive local tissue destruction together with a chronic systemic inflammatory response with potential for damaging distant organs such as the brain.

The brain was originally considered an immunoprivileged microenvironment due to the existence of the BBB; however, it is now recognized that the BBB is incomplete in both the circumventricular organs and the choroid plexus regions [36–38]. The incomplete BBB provides an opportunity for systemic proteins and cells to gain access to the CNS. Microglial cells in the circumventricular organs have been demonstrated to express the CD14 receptor and the toll-like receptor 4 (TLR-4), suggesting that these cells are capable of detecting bacterial PAMPs [37, 39, 40].

This initial concept received additional support from clinical studies that demonstrated a significant correlation between tooth loss due to PD and memory loss in AD [16]. The same researchers reported that individuals with deteriorating memory also have increased incidence of the apolipoprotein E (ApoE) allele 4 [41]. ApoE is a cholesterol-transporting protein and, in the brain (with a few exceptions), is synthesized largely by astrocytes for repair of and protection of neurons [42]. AD individuals are known to have antibodies to oral bacteria in their plasma along with an increased presence of TNF-α [43, 44]. It was also reported that a high titer of circulating IgG from a range of PD pathogens, during advancing age, statistically correlates with a possible onset of mild cognitive impairment and AD [33]. Methodological studies demonstrating the presence of bacteria within the cerebral tissues are sparse. The limiting factor may be availability of suitable postmortem (PM) tissue and corresponding data regarding cognitive impairment and PD in relation to pathogens such as *Treponema denticola, Tannerella*

forsythia, and *Porphyromonas gingivalis* indigenous to the oral cavity. One seminal study using molecular and immunological methodologies demonstrated the presence of seven oral *Treponema* species in 14 of 16 AD cases, reaching statistical significance [45]. Moreover, immune-suppressed rodents demonstrated an increased risk from endodontic infections with the fastidious oral spirochete *T. denticola* [46]. Thus it is plausible that bacteria and/or their virulence factors have a greater chance of accessing the brain of individuals with AD due to their immuno-compromised status. The aim of this study was to determine if the major PD bacteria (*T. denticola, T. forsythia,* and *P. gingivalis*) and/or bacterial components are present in brain tissue of individuals with and without dementia.

MATERIALS AND METHODS

Human brain specimens and tissue sectioning

All research procedures met approval of our academic institute (Ref No. 071) and the ethical guidelines, including adherence to the legal requirements of study in the UK. PM human brain tissue was obtained from the Brains for Dementia Research network and was provided by the Newcastle Brain Tissue Resource. These specimens included previously diagnosed AD ($n = 10$) and, where possible, age-matched non-AD control ($n = 10$) brains. Samples of frozen human brain tissue from an area adjacent to the lateral ventricle of the parietal lobe were dissected using aseptic methods. Precautions were taken to prevent cross contamination during sample preparation. The brain specimens (1 cm^3 core) were in sterile polystyrene tubes in dry ice when received via next-day-delivery courier service. The PM interval for all AD cases ranged from 4 to 12 h and the non-AD age-matched control brains from 16 to 43 h (Table 1). On receipt, all specimens were allocated a code number and thereafter all data recorded about those cases were identified by that code. The experimenter was completely unaware of which cases corresponded to AD and control brains. The cases are identified here as being AD and non-AD controls for clarity of reporting. A 3-mm^2 section of the brain tissue was separated from the original snap-frozen unfixed cores and mounted onto a specimen holder using the OCT® adhesive (Fisher Scientific). Sections (10 μm thickness) were cut using the Leica CM1850 cryostat (Leica, UK) and were collected onto Superfrost +® glass slides (Leica, UK). The sections were used immediately or stored at –80°C until needed.

Table 1
The age and postmortem interval of the cases analyzed

Case	Age	Postmortem interval (h)	LPS detected
AD 1	78	12	No
AD 2	77	8	No
AD 3	84	8	Yes
AD 4	84	8	No
AD 5	85	9	Yes
AD 6	83	9	No
AD 7	80	4	No
AD 8	83	10	Yes
AD 9	63	11	No
AD 10	83	12	Yes
Non-AD 1	69	16	No
Non-AD 2	72	17	No
Non-AD 3	103	21	No
Non-AD 4	78	23	No
Non-AD 5	89	24	No
Non-AD 6	81	43	No
Non-AD 7	78	34	No
Non-AD 8	89	34	No
Non-AD 9	67	22	No
Non-AD 10	22	22	No

In vitro culture of SVGp12 cells

The SV40 immortalized normal human glial cell line SVGp12 was obtained from the American Type Culture Collection ATCC Ref No. CRL-8621 (Manassas, VA, USA) and cultured in Eagle's minimal essential medium supplemented with heat-inactivated 10% fetal calf serum, 4 mM glutamine, 2 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen) without the addition of penicillin/streptomycin. Cells were cultured in flasks (T25, T75) or on sterile uncoated glass coverslips placed in six well plates in the presence of appropriate culture medium and incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air with regular media changes every two to three days where applicable.

In vitro responses of the SVGp12 cell line to *P. gingivalis* ATCC 33277 was examined following initial confirmation of LPS in culture supernatant (Table 2). SVGp12 cells were exposed for 24–48 h to diluted *P. gingivalis* culture supernatant.

Immunofluorescence labeling of brain tissue sections

Tissue sections from snap-frozen brain were allowed to air dry at room temperature and stabilized for 5 min in cold analar-grade acetone (Fisher Scientific, UK). Unless otherwise stated, no quenching of autofluorescence or any other antigen retrieval step was employed. Sections were equilibrated in 0.01 M

Table 2
Source of antibodies and their working concentration and/or dilutions applied

Antibody	Source	Final concentration and/or dilution
Mouse anti-CD14 (clone HCD14)	Thermo-Fisher	4 μg/ml
Mouse anti-*P. gingivalis* (Clones 1B5 and 1A1) tissue culture supernatant	Prof. M. A. Curtis (co-author)	1B5 1/10, 1A1 1/50
Mouse anti-*P. gingivalis* (Clone 61BG1.3) tissue culture supernatant	Prof. R. Gmur, University of Zurich, Switzerland	Neat and 1/5
Rabbit anti-*T. forsythia* (rBspA)	Dr A Sharma, State University of New York at Buffalo, NY, USA	1/50
Mouse anti-*T. denticola*	Tissue culture supernatant raised in-house from hybridoma cell lines TDII (HB-9966) and TDIII (HB-9967) purchased from ATCC	Neat and 1/5
Blocking solution	In-house: 0.01 M phosphate buffered saline (PBS) pH 7.3 containing 0.01% normal goat serum and 2.5% tween 20	-
Normal goat serum (X0907) and normal rabbit serum (X0902)	DakoCytomation, Germany,	0.01%
E. coli LPS	Sigma Aldrich, UK	4 μg/μl

phosphate buffered saline (PBS) once for 5 min and blocked in PBS containing 0.01% normal goat or rabbit serum and 2.5% tween 20. The sections were incubated overnight at 4°C in the following monoclonal antibodies raised to different epitopes of *P. ginigvalis*: where clone 1B5 of anti-*P. gingivalis* detects both LPS and gingipains [47] and clone 1A1 [48] and 61BG1.3 [49] recognize gingipains specifically from this bacterium (Table 2). Anti-*T. forsythia* antibodies (recombinant bacterial surface protein A (rBspA), gift from Dr. Ashu Sharma, USA), were raised against the rBspA protein which was heat/SDS denatured before immunization in rabbits [50]. Anti-*T. denticola* antibodies were raised in-house from hybridoma cell lines (TDII (HB-9966) and TDIII (HB-9967) from ATCC) according to the manufacturer's instructions. In addition, anti-CD14 (Fisher Scientific, UK) was also applied to tissue sections following dilution in the blocking solution (Table 2). The secondary detection was carried out using either the goat anti-mouse or the goat anti-rabbit IgG conjugated to FITC (Sigma-Aldrich, UK) at 5 μg/ml. Following further washes in PBS for three times 5 min, sections were mounted under a glass coverslip using propidium iodide (Vector Laboratories, Peterborough, UK). Labeling was observed and images were captured using the 510 series Zeiss confocal microscope (Carl Zeiss Ltd).

Immunofluorescence labeling of SVGp12 cells

SVGp12 cells were immunolabeled following fixation of cells (on coverslips) in 10% neutral buffered formalin ranging from 1 h to overnight at 4°C and subsequently washed in 0.01 M PBS, pH 7.3. Primary antibodies [mouse anti-CD14 and anti-*P. gingivalis* (clones 1B5 and 1A1) (Table 2)] were applied to cells in the blocking solution (Table 2) and the conditions for incubation and secondary detection was performed as described for labeling of brain tissue sections above.

Controls

The primary antibody was either omitted from all control brain tissue sections and from cells on coverslips or included anti-*P. ginigvalis* (clones 1B5 and 1A1) antibodies on medium-control-challenged cells.

Bacteria and LPS

P. gingivalis (ATCC 33277 and W50) was grown for 48 h, in a brain/heart-infusion broth supplemented with haemin (5 mg/l), and menadione (1 mg/l), purchased from Sigma-Aldrich, (UK). Following growth, each culture was centrifuged at 15,000 rpm at 4°C for 30 min to pellet bacterial cells and the culture supernatant was collected. Aliquots (1 ml or 0.5 ml) were prepared in pre-labeled sterile Eppendorf® tubes and stored at −80°C until needed. Protease inhibitors (cOmplete ULTRA®, Roche Applied Science, USA) were added to one of the aliquots, from the culture supernatants and the growth medium (control) and freeze dried for at least 12 h. The lyophilized powder was re-suspended in a 200 μl volume of lysis buffer containing 50 mM Tris pH 8.0, 1% NP40, 150 mM NaCl, and 5 mM EDTA before the total protein concentration was determined. These aliquots were stored at −20°C until needed. Commercially prepared (phenol extracted) lyophilized powder from *Escherichia coli* LPS was obtained from Sigma-Aldrich (UK) and re-suspended (1 mg) in 250 μl lysis buffer containing protease inhibitors (used above) and stored at −20°C.

Positive and negative control cell lysates

Following exposure to either the sterile bacterial growth medium (medium control) or to the *P. gingivalis* culture supernatant, SVGp12 cells were pelleted and washed twice in cold sterile PBS with centrifugation (5 min at 2,500 rpm). The cells were lysed in buffer containing protease inhibitors (used above). Following incubation on ice for 30 min and frequent vortex mixing, the cell homogenate was centrifuged at 12,000 rpm for 20 min at 4°C in a microcentrifuge. The supernatant was collected in pre-labeled tubes and stored at −20°C.

Human brain tissue lysates

To prevent secondary cross contamination of the human brain during the experimental procedures, the specimens were handled only in the bench-top microflow cabinet (Astec Microflow Ltd., UK), which is regularly serviced and was always disinfected with 2% sodium hypochlorite solution (Fisher Scientific) and sprayed with 70% ethanol before use and at the end of the experiment. The experimenter wore disposable face masks and gloves when handling tissue and preparing the tissue lysate. A 3-mm^2-thick section of all brain specimens was taken from the original snap-frozen unfixed tissue core and minced in the lysis buffer containing protease inhibitors as above. Following incubation on ice for 30 min and vortex mixing, the tissue homogenate was centrifuged and collected in pre-labeled tubes and stored at −20°C. The total protein concentration of all lysates was determined using a colorimetric assay. Protein concentration was obtained from a standard curve prepared using 100–400 μg/ml bovine serum albumin diluted in lysis buffer containing protease inhibitors. After Coomassie® protein assay reagent (Sigma-Aldrich, UK) was added to all standards and test samples, absorbance was measured at 595 nm wavelength using the Jenway 7315 spectrophotometer. The concentration of the unknowns was calculated by comparing absorbance values with the standard curve.

Immunoblot analysis

To confirm the presence of LPS and gingipains in *P. gingivalis* culture supernatant and medium control (initially at 60 μg, later adjusted to 30 μg per lane), electrophoresis was performed under reducing conditions using 12.5% (w/v) SDS-PAGE gels. The proteins were electro-transferred to a polyvinylidene difluoride membrane (PVDF, Immobile-P; Millipore, UK) and blocked for 30 min at room temperature in 5% (w/v) skimmed milk/PBS prior to incubation overnight at 4°C with the primary anti-*P. gingivalis* antibodies (clones 1B5 and 1A1) diluted 1/20 and 1/50 respectively, in 5% (w/v) skimmed milk/PBS. Following three 15-min washes in PBS containing 0.2% tween 20, the membrane was incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig secondary antibody (Chemicon) diluted 1/10,000 in 5% (w/v) skimmed milk/PBS for 2 h at room temperature. Following further washes in PBS/tween 20, (3x15 min each) the bands were detected using the enhanced chemiluminescence detection reagent (Bio-Rad, UK) under transmitted ultra violet light in a gel-doc imaging station using the Molecular Analyst software (Bio-Rad, UK). India ink was used to stain the membrane to determine the amount of protein transferred onto the membrane(s) as a loading control. Electrophoresis of brain tissue and SVGp12cell samples was also carried out under reducing conditions as described above, except the extracts loaded were 30 μg per lane. Electrophoresis and immunoblotting were performed at least six times and cross checked by at least two experimenters.

Statistical analysis

The significance of the difference between AD and non-AD controls was analyzed by a non-parametric Mann-Whitney U test for two independent samples (IBM SPSS statistics 20). The differences were considered significant at $p \leq 0.05$.

RESULTS

Immunofluorescence labeling

Controls

All control tissue sections from the human brain were exposed to the same conditions as the test sections. The sections in which the primary antibody was omitted remained negative with the FITC-labeled secondary detection system (Figs. 1a, 2a (phase overlay), 3a). Some generalized autofluorescence was associated with erythrocytes, but remained below the threshold of the noise-to-signal ratio except for the elastin in arteries. Strong autofluorescence was associated with brain pigment, but this was of a different wavelength and color to that of the FITC signal. SVGp12 cells challenged with medium control remained negative when incubated with the anti-*P.*

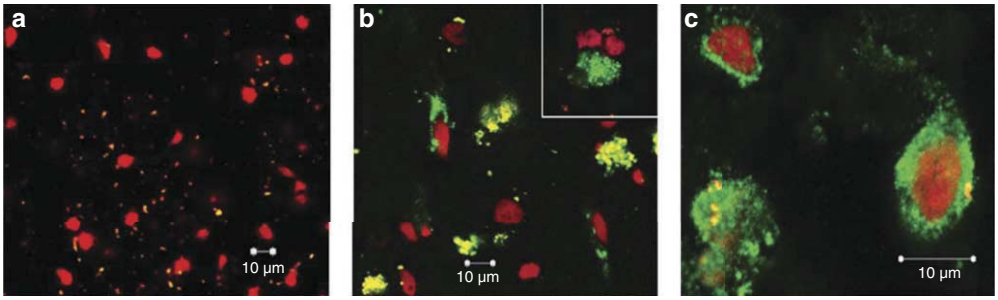

Fig. 1. Human AD brain. Confocal microscope images captured from snap-frozen brain tissue sections from Alzheimer's disease (AD) showing nuclei due to propidium iodide (PI) uptake. The images are overlaid with PI and the FITC signals. a) Negative control, primary antibody omitted. b) Immunolabeled using the anti-*P. gingivalis* (clone 1B5) antibody overnight at 4°C followed by detection using goat anti mouse FITC. Insert shows extracellular aggregates with granular (pebbly) appearance embedded within a smoother matrix. c) An adjacent section from the same brain labeled with mouse anti-CD14 for surface membrane labeling.

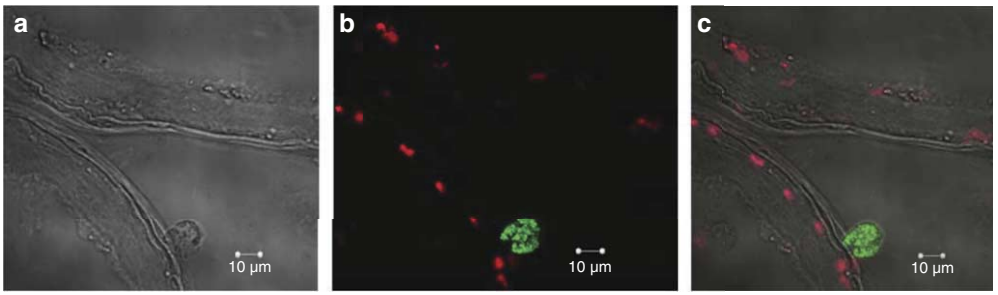

Fig. 2. An arterio-venous sinus. Immunolabeling as described for Fig. 1. a) Phase contrast image with the extracellular aggregate within the lumen of the arterio-venous sinus. b) The extracellular aggregate is labeled with the anti-*P. gingivalis* antibody (clone 1B5). c) The phase contrast image from (a) is overlaid on the immunofluorescent image from (b).

gingivalis antibody clones 1B5 (Fig. 4a) and 1A1 (not shown) and when the primary antibody was omitted.

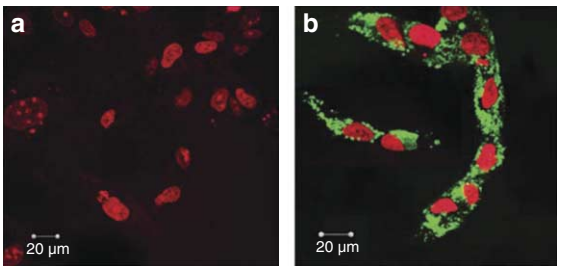

Fig. 3. SVGp12 cells challenged with *P. gingivalis* culture supernatant. SVGp12 cells exposed to medium control and *P. gingivalis* culture supernatant for 24 h. Immunolabeling (anti-*P. gingivalis*, 1B5) and nuclear stain are as for Fig. 1. a) The cells exposed to medium control remained negative despite the application of the antibody. b) Cells exposed to the *P. gingivalis* culture supernatant demonstrated intense labeling localized to membrane-bound vesicles.

Human brain tissue sections

Post labeling the human brain tissue sections with the mouse anti-*P. gingivalis* (clone 1B5), revealed strong cellular surface membrane labeling in four out of 10 AD cases (Fig. 1b) and not in the non-AD age-matched controls. Extracellular aggregates "pebbly" or "granular" in appearance were also present and were intensely labeled in the same four AD cases (Fig. 1b insert). Surface membrane labeling was validated with a monoclonal anti-CD14 antibody in adjacent brain test sections (Fig. 1c). The extracellular aggregates were frequently observed within the brain parenchyma and in association with arterio-venus sinuses (Fig. 2b) as clearly shown by a phase image overlaid on the immunofluorescence image (Fig. 2c). No labeling associated with anti-*P. gingivalis* antibodies (clones 1A1 and 61BG1.3) was observed in any of the tissue sections from control and/or AD brains. No immunolabeling was observed with the anti-*T. forsythia* antibodies raised to rBspA protein nor with the anti-*T.*

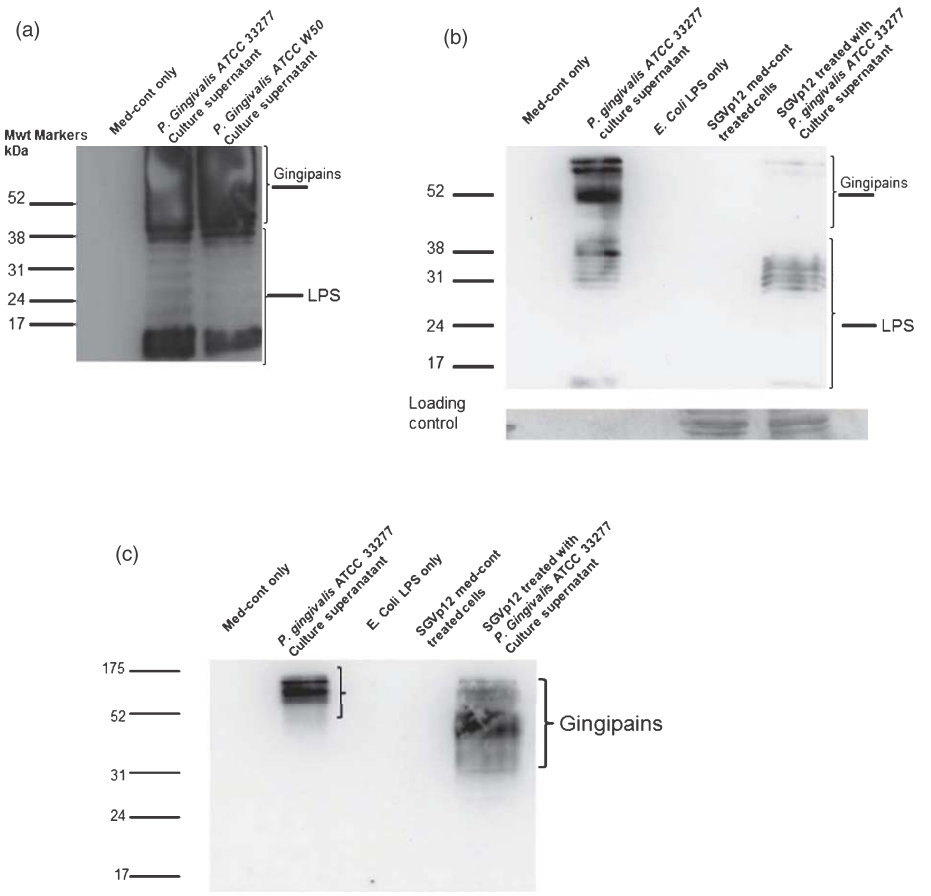

Fig. 4. Immunoblots to demonstrate gingipains and LPS are components of the culture supernatant from *P. gingivalis* ATCC 33277 and W50. Total protein/lane (60 μg) was loaded on a 12.5% SDS-PAGE gel followed by a successful transfer to a PVDF membrane. Immunoblotting using the primary antibody (anti-*P. gingivalis* clone 1B5) and secondary detection using goat anti-mouse conjugated to HRP (see text). a) Medium control (lane 1) failed to produce any bands whereas the positive control culture supernatants from *P. gingivalis* ATCC 33277 (lane 2) and W50 (lane 3) demonstrated an abundance of gingipains (dark long band above and below 52 kDa) and a number of bands (45–12 kDa) corresponding to LPS in *P. gingivalis* culture supernatants from ATCC 33277 and W50. b) Total protein/lane (30 μg) was loaded on the gel as in Fig. 4a. The same medium control (lane 1) failed to produce any bands, whereas the positive control culture supernatant (lane 2) demonstrated bands for gingipains at the higher molecular weight and at 45–12 kDa corresponding to LPS in *P. gingivalis* culture supernatant from ATCC 33277. *E. coli* LPS (lane 3), and cells treated with medium control (lane 4) showed no bands. The result in lane 5 confirmed the de-novo antigen detected by the anti-*P. gingivalis* (clone 1B5) antibody was LPS on SVGp12 cells. The loading control represented by India ink failed to stain the medium control (lane 1), culture supernatants (lanes 2 and 3), and *E. coli* LPS (lane 3). c) A duplicate blot to that shown in (b) was exposed to the anti-*P. gingivalis* clone 1A1 antibody. The same medium control (lane 1) produced no bands, whereas the positive control culture supernatant from ATCC 33277 (lane 2) demonstrated bands for gingipains at the higher molecular weight size. *E. coli* LPS (lane 3) and the cells treated with medium control (lane 4) failed to produce any bands. The presence of gingipains in SVGp12 cells was confirmed (lane 5).

denticola antibodies, although they weakly labeled the whole bacterial cells (not shown). Experiments using these antibodies were terminated at this stage.

In vitro culture of SVGp12 cells challenged with P. gingivalis culture supernatant

Immunolabeling using the anti-*P. gingivalis* (clone 1B5) antibody demonstrated that the surface membrane of SVGp12 glial cell line was intensely labeled and appeared highly vesiculated (Fig. 3b). The anti-*P. gingivalis* antibody (clone 1A1) which is specific for gingipains was applied to SVGp12 treated cells with *P. gingivalis* ATCC 33277 for over 24 h and demonstrated that the labeling was restricted to perinuclear sites and in lysosomes (not shown).

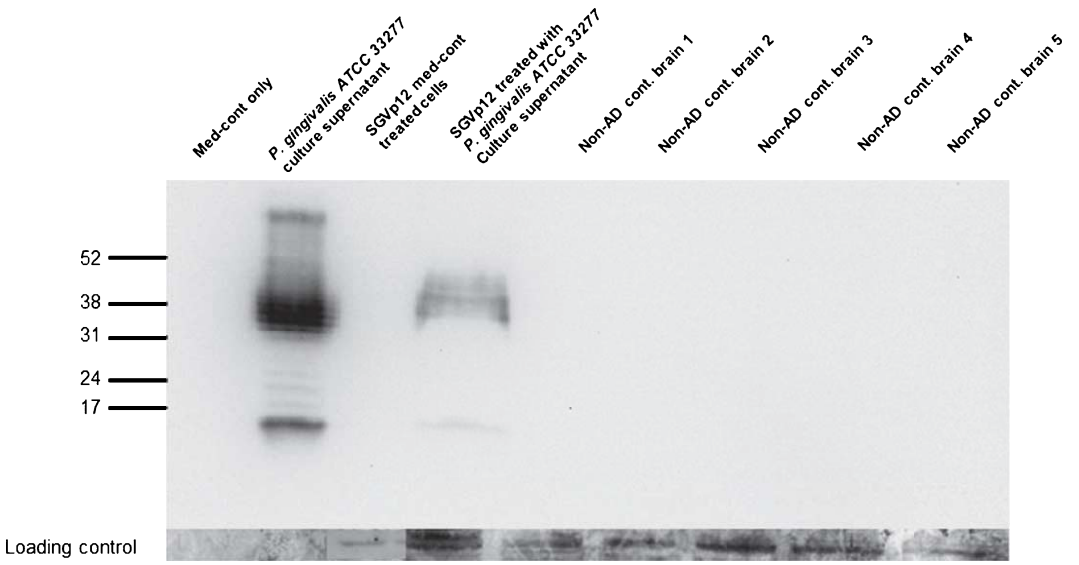

Fig. 5. Human non-AD control brain tissue immunoblotted with anti *P. gingivalis* (clone 1B5). Electrophoresis and protein transfer was as for Fig. 4. Total protein was 30 μg per lane and, unless otherwise stated, conditions for immunoblotting and loading control were as described in Fig. 4b. While the negative controls (lanes 1 and 3) and positive controls (lanes 2 and 4) remained as expected, there were no bands in the specimens from all five non-AD control brains (lanes 5–9).

Immunoblot analysis

LPS and gingipains were components of P. gingivalis culture supernatant

The medium control (sterile liquid medium) analyzed under reducing conditions using immunoblotting with the anti-*P. gingivalis* (clone 1B5) antibody (Fig. 4a) failed to show any bands (lane 1). The lanes with culture supernatants from *P. gingivalis* ATCC 33277 (lane 2) and W50 (lane 3) both showed a dark, high molecular weight band for gingipains (Fig. 4a) and a ladder of bands around 45–12 kDa corresponding to LPS (Fig. 4a). These data agree with the previously published literature for W50 LPS using the same antibody [47, 51].

Positive and negative controls

All control samples were analyzed using immunoblotting with the anti-*P. gingivalis* (clones 1B5 and 1A1) antibodies (Fig. 4a-c); no bands were visible in lanes loaded with the medium control (lane 1), *E. coli* LPS (lane 3, Fig. 4b-c), and SVGp12 cells treated with sterile medium control (lane 4, Fig. 4b-c). A ladder of bands in the range of 45–12 kDa, corresponding to LPS, was detected in the *P. gingivalis* culture supernatant (lane 2, Fig. 4a-b) and SVGp12 cells challenged with the same supernatant for 48 h (lane 5, Fig. 4b). Only high molecular weight bands were observed with anti-*P. gingivalis* (clone 1A1) in both the culture supernatant (lane 2, Fig. 4c) and SVGp12 cells challenged with the same supernatant (lane 5, Fig. 4c). Medium control (lane 1), *P. gingivalis* culture supernatant (lane 2-Fig. 4a-b, 5–7), and *E. coli* LPS (lane 3-Fig. 4b-c and 5–6) consistently failed to stain with India ink.

Human control brain

Immunoblotting with anti-*P. gingivalis* (clone 1B5) (Fig. 5) detected no bands in lanes corresponding to the sterile medium control (lane 1) and SVGp12 cells treated with sterile control medium (lane 3). A laddering pattern of bands (45–12 kDa) corresponding to LPS was observed in both the *P. gingivalis* culture supernatant (lane 2) and in SVGp12 cells treated with the same culture supernatant (lane 4). However, no bands were detected in the lanes loaded with the age-matched non-AD control brains labeled 1–5 (Fig. 5, lanes 5–9). Further control brains (Non-AD 6–10) were also analyzed on a separate blot under identical conditions and again all of the test lysates (from non-AD brains 6–10) remained negative (data not shown).

AD brain

Consistently, no bands (Fig. 6) corresponding to the sterile medium control (lane 1), *E. coli* LPS (lane 3),

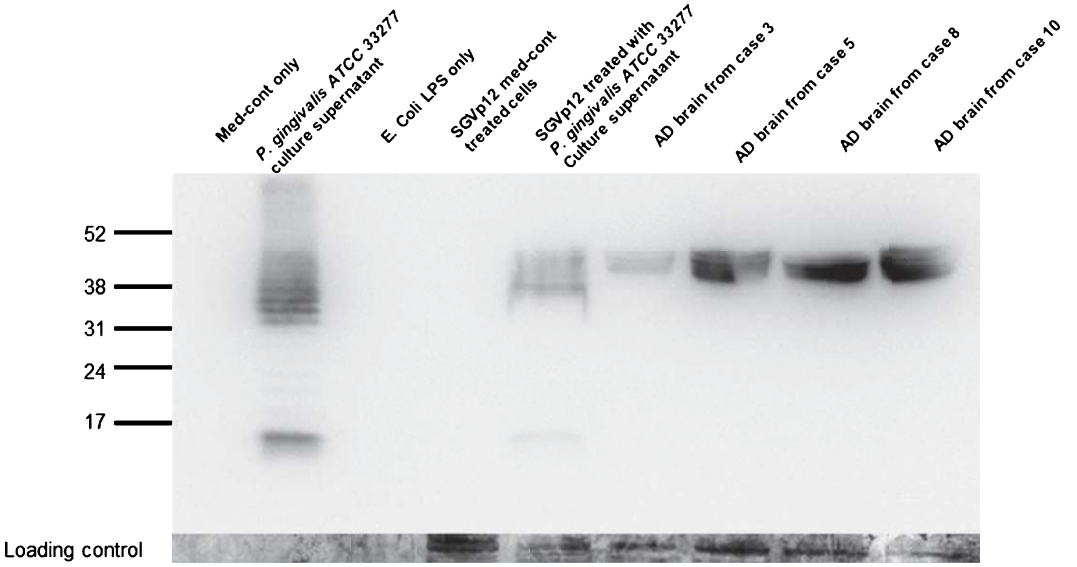

Fig. 6. Human AD brain tissue immunoblotted with anti-*P. gingivalis* (clone 1B5). Electrophoresis and protein transfer was as for Fig. 4. Total protein/lane, immunoblotting reagents, and loading control conditions were the same as for Fig. 4b. The orders of negative and positive controls (lanes 1–5) are as for Fig. 4b. Anti-*P. gingivalis* antibody (1B5) detected bands characteristic of the LPS at the expected molecular weight in AD case numbers 3, 5, 8, and 10.

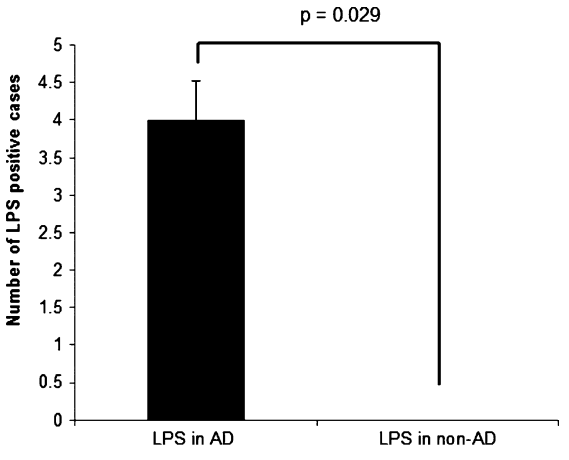

Fig. 7. Statistical analysis. The non-parametric Mann-Whitney U test for two independent samples (IBM SPSS statistics 20) confirmed there was statistical difference in AD compared with non-AD cases ($p = 0.029$).

and SVGp12 cells treated with sterile medium control (lane 4) were detected following incubation in the anti-*P. gingivalis* (clone 1B5) antibody. Bands in a characteristic *P. gingivalis* LPS laddering pattern were observed in lanes loaded with *P. gingivalis* culture supernatant (lane 2), SVGp12 cells treated with the culture supernatant (lane 5) and in AD cases designated 3, 5, 8, and 10 (lanes 6–9) between 45–12 kDa molecular weight positions (Fig. 6). The AD cases designated 1, 2, 4, 6, 7, and 9 were negative by immunofluorescence but when tested by immunoblotting under identical conditions to those described for Fig. 6, they (AD cases 1, 2, 4, 6, 7, and 9) consistently failed to detect any bands (data not shown).

Statistical analysis

Immunolabeling and immunoblotting using the anti-*P. gingivalis* (clone 1B5) antibody identified four out of 10 of the AD brain specimens as being positive while 10 out of 10 non-AD age-matched controls were negative for LPS. The non-parametric Mann-Whitney U test demonstrated that the four positive AD cases were statistically significant ($p = 0.029$) compared with the non-AD controls (Fig. 7).

DISCUSSION

The theory of the human mouth as a focus of infection states that oral microbial infections contribute to the developing pathologies of remote body organs by infiltrating into the systemic system [52, 53]. This concept prompted us to explore the hypothesis in relation to finding a causal link between PD and AD. Studies to understand the relationship between environmental factors such as pathogens and their role in dementia

including the deposition of Aβ are crucial to understanding the contribution made by microbial agents to disease pathogenesis and progression. An investigation of the etiological hypothesis will therefore rely on sampling tissues from PM specimens obtained from AD and non-AD individuals, with and without evidence of oral infection, and from older subjects with longer interval between onset of dementia and death. All these variables can be investigated once autopsy contamination of tissues from anaerobic periodontal pathogens in the oral cavity and the CNS in PM specimens has been excluded. Potentially important bacteria include *P. gingivalis*, *T. denticola*, and *T. forsythia*, one of which (*T. denticola*) has already been linked to neurodegeneration and dementia [20, 45].

We assessed the presence of the major periodontopathogenic bacteria *P. gingivalis*, *T. denticola*, and *T. forsythia*, in a small series of 10 AD brains with a 12 to 24 h PM delay and 10 non-AD cases with an extended (16 to 43 h) PM delay. As stated (in the Materials and Methods section), a number of antibodies were tested on the AD and non-AD age-related control sections using indirect immunofluorescence. The *T. forsythia* [50] and *T. denticola* antibodies poorly detected the native antigen on whole cells and in the brain tissue sections. Hence, further assessment of these organisms was not pursued. Anti-*P. gingivalis* antibodies, on the contrary, intensely labeled *P. gingivalis* whole cells and their antigen within tissue sections. This prompted further investigation of this organism in the brain tissue of individuals with dementia with a validated neuropathological diagnosis of the sporadic form of AD (c/o Brains for Dementia Research).

The monoclonal antibody used in this investigation is well characterized [47] and is specific for *P. gingivalis* LPS and gingipain epitopes [51]. To delineate if it was the LPS and/or gingipains that were being detected on the surface of cells by the anti-*P. gingivalis* (clone 1B5); two additional and specific monoclonal antibodies to gingipains [48, 49] were also used. Immunofluorescence labeling of cells and the immunoblot analysis conclusively revealed that it was LPS and not gingipains from *P. gingivalis* that was detected in AD brain specimens. The same antibody confirmed that the culture supernatant from *P. gingivalis* ATCC 33277 contained LPS, thus supporting the previously published literature from *P. gingivalis* W50 [47, 51]. The non-parametric Mann-Whitney U test demonstrated that, even from this small series, AD cases provided a statistically significant result ($p = 0.029$) compared with the non-AD controls. A number of researchers have found bacteria [54] and viruses associated with Aβ deposits and tau positive neurofibrillary tangles [17–20] in the late-onset AD brains. However, we only detected the *P. gingivalis* LPS epitope on glial cells which participate in the innate immune responses in relation to infection in the brain.

These results indicate that the brain of AD patients is at a greater risk of secondary chronic infection from the periodontal pathogen *P. gingivalis* which has long been implicated in chronic and severe adult periodontitis [55, 56]. Dental records of the individuals whose brain specimens we examined were not available; hence, it is difficult to comment on any direct relationship of PD with AD during life. However, due to the poor memory exhibited by AD patients, these individuals may forget to maintain optimal oral hygiene which during advanced stages of AD would be expected to deteriorate even further [57–60].

Bacteremia in AD patients is inevitable because of impaired swallowing reflexes during the late stages of the disease process. The impaired functionality of the muscles associated with swallowing is likely to increase oral pathogens gaining entry into the systemic circulation. Direct access of pathogens and/or their endotoxins into the CNS from the circumventricular organs can take place because these regions of the brain have an incomplete BBB [36, 38] and are the primary port for bacterial and LPS entry into the brain following systemic infections [37]. An alternative route of direct access of bacteria and/or their products into the CNS is from the perivascular space using systemic circulation.

Multiple systemic infections are reported to exacerbate premorbid cognitive status in AD patients and the current view indicates that this is the result of proinflammatory mediators crossing the BBB [43, 61, 62]. We frequently observed aggregates of "LPS" within the brain tissue as well as in some intravenous sinuses. Detecting systemic LPS is relevant because it is a powerful stimulator of the innate immune system. Once in the brain it will activate local glia to mount an innate immune response. The LPS hyper-sensitized microglia increase synthesis of inflammatory mediators, such as TNF-α, IL-1β, and IL-6, complement factors, TLRs 2 and 4 and nitric oxide that release free radicals and reactive oxygen species [24] and increase tissue damage.

In this study, the *in vitro* data has demonstrated that SVGp12 cells adsorbed LPS from *P. gingivalis* culture supernatant that contained a battery of molecular determinants, including endotoxin (LPS) and extracellular cysteine proteases (gingipains) [63, 64] as well as metabolites such as butyric and propionic acids.

Of these, LPS was adsorbed on the surface membrane by the astroglial cell line whereas gingipains demonstrated an intracellular localization [65]. This observation supports the results from the human brain which demonstrated that LPS was adsorbed by CNS glia as detected by the surface membrane immunolabeling using the anti-*P. gingivalis* monoclonal antibody [47, 51], and validated by the anti-CD14 receptor antibody. In addition, the immunoblot detecting characteristic LPS laddering pattern using the same (anti-*P. gingivalis*) antibody [47, 51] on the same AD cases unequivocally demonstrates that it was LPS adsorbed by CNS glia in the human brain. LPS was absent from the control brain tissues with PM interval extending to 43 h.

In summary, immunolabeling and immunoblotting of brain tissue from individuals with and without dementia has provided statistically significant evidence to implicate the presence of LPS from *P. gingivalis* in AD cases with 12 h maximum PM delay. No evidence of LPS from *P. gingivalis* was detected in the non-AD control tissues with longer PM delay (up to 43 h). Once in the brain, microglia will respond to the LPS and activate the CNS innate immune system. This will result in the initiation of a pro-inflammatory cascade to bring about the local release of potentially neurotoxic substances such as cytokines, complement factors, and reactive oxygen species and exacerbate the preexisting disease-related inflammatory pathology.

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Active Invasion of *Porphyromonas gingivalis* and Infection-Induced Complement Activation in ApoE$^{-/-}$ Mice Brains

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Abstract. Periodontal disease is a polymicrobial inflammatory disease that leads to chronic systemic inflammation and direct infiltration of bacteria/bacterial components, which may contribute to the development of Alzheimer's disease. ApoE$^{-/-}$ mice were orally infected ($n = 12$) with *Porphyromonas gingivalis, Treponema denticola*, *Tannerella forsythia*, and *Fusobacterium nucleatum* as mono- and polymicrobial infections. ApoE$^{-/-}$ mice were sacrificed following 12 and 24 weeks of chronic infection. Bacterial genomic DNA was isolated from all brain tissues except for the *F. nucleatum* mono-infected group. Polymerase chain reaction was performed using universal 16 s rDNA primers and species-specific primer sets for each organism to determine whether the infecting pathogens accessed the brain. Sequencing amplification products confirmed the invasion of bacteria into the brain during infection. The innate immune responses were detected using antibodies against complement activation products of C3 convertase stage and the membrane attack complex. Molecular methods demonstrated that 6 out of 12 ApoE$^{-/-}$ mice brains contained *P. gingivalis* genomic DNA at 12 weeks ($p = 0.006$), and 9 out of 12 at 24 weeks of infection ($p = 0.0001$). Microglia in both infected and control groups demonstrated strong intracellular labeling with C3 and C9, due to on-going biosynthesis. The pyramidal neurons of the hippocampus in 4 out of 12 infected mice brains demonstrated characteristic opsonization with C3 activation fragments ($p = 0.032$). These results show that the oral pathogen *P. gingivalis* was able to access the ApoE$^{-/-}$ mice brain and thereby contributed to complement activation with bystander neuronal injury.

Keywords: Alzheimer's disease, chronic periodontitis, inflammation, periodontal bacteria

INTRODUCTION

Alzheimer's disease (AD) is a form of dementia associated with cognitive decline and irreversible memory loss. The pathological hallmarks of AD brains are an accumulation of intracellular hyperphosphorylated tau-positive neurofibrillary tangles (NFT) together with insoluble, fibrillary amyloid-β (Aβ) plaques, which are traditionally recognized as being triggers that stimulate glial cell activation and initiate local innate immune responses [1]. AD has a complex etiology in which the genetic makeup of the individual and environmental factors play a role. The late-onset form of AD is particularly interesting as its etiology remains unknown despite the known genetic risk factors, including apolipoprotein E (ApoE) gene and its E4 allele inheritance [2, 3]. This risk factor is

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associated with severe AD pathology and an enhanced inflammatory response by microglia [4].

Peripheral infections also serve as a significant risk factor affecting mental health as demonstrated in clinical studies in which cognitive decline and deteriorating memory are reported [5–7]. A range of infective agents is consistently being linked to AD [8], including viruses such as the Herpes simplex virus type 1 (HSV-1) [9]; bacteria such as *Chlamydophila pneumoniae* (*C. pneumoniae*) [10]; and various types of spirochetes, including *Borrelia burgdorferi* (*B. burgdorferi*) [11–13] and periodontal *Treponema* spp., [14] and more recently *Porphyromonas gingivalis* (*P. gingivalis*) [15]. *P. gingivalis* and some oral *Treponema* species are invasive and virulent within their original niche where they induce gingival inflammation that leads to connective tissue degradation and alveolar bone resorption around teeth [16, 17]. Once the junctional epithelium that links the gingiva to the tooth enamel transforms to pocket epithelium, pathogenic bacteria induce bacteremia and initiate systemic inflammation by infiltrating the local blood vessels [18–20]. These factors may lead to various chronic inflammatory disorders such as cardiovascular disease(s) [21, 22], diabetes [23], rheumatoid arthritis [24–26], premature births [27], and AD [14, 15, 28, 29].

Clinical studies by Stein et al. [28] support a strong association between tooth loss due to periodontal disease and the development of AD. They noted a greater rate of cognitive decline occurring in carriers of the ApoE $\varepsilon 4$ allele variant with fewer teeth [30]. Although chronic infection by *Treponema pallidum* is widely accepted for the atrophic form of general paresis, it and *B. burgdorferi* infections (etiological bacteria for Lyme disease) are also reported to result in dementia [11–13]. These spirochete infections give rise to the similar pathological hallmark features such as Aβ4 plaques and NFTs seen in AD [11–13]. This is regarded as a direct link between spirochete infections and the development of AD. *C. pneumoniae* and HSV-1 infections of the brain also appear to be associated with the Aβ deposition observed in AD [9, 10, 12]; however, their role as infection by individual pathogen or occurring as co-infections with the invading spirochetes remains under investigation [12]. *T. denticola* and *P. gingivalis* oral infections of the brain are also reported [14, 15], but their direct involvement with the deposition of Aβ4 and NFTs is not clear.

Inflammation in the brain is characterized by the presence of reactive microgliosis and astrocytosis (inflammatory phenotype) and is an accepted component of AD pathology [1]. Traditionally, the inflammatory component of the pathology in AD is believed to be the result of cytokines, oxidative stress, and complement activation, including the membrane attack complex due to the hallmark proteins of AD [1]. However, the fact that pathogens are implicated in some forms of central nervous system (CNS) diseases that result in the eventual development of AD [11–13], suggests that the existing hypothesis cannot exclude a possible role of chronic infections generating an inflammatory pathology in AD. Concerning chronic infections in AD brains, in 2008 two independent research groups implicated the indirect role of periodontal pathogens and/or their virulence factors in the development of AD [31, 32] involving acute-phase proteins, including cytokines, as a plausible link between periodontal bacteria and inflammatory AD pathology. Miklossy (2008) proposed a direct link between oral spirochetes and AD via bacterial infection of the brain in which either the spirochetes or their virulence factors activate the classical and the alternative pathways of complement, resulting in vital cell loss via the membrane attack complex [33]. Thus, the presence of cytokines and/or an activated complement cascade can be used as a marker to measure CNS inflammation in this context.

Further demonstration of a high titer of antibodies against periodontal pathogens in the serum of elderly who progressed to AD also suggests the possible association between periodontal disease and AD [34].

Poor oral hygiene [35] is strongly linked to the development of dementia; however to date there are very few reports establishing an experimental link between periodontal disease and AD. Two studies using human brain tissue explored the impact of periodontal infections on AD [14, 15]. These studies examined AD brain tissue specimens using molecular profiling methodologies to identify seven *Treponema* species [14] and the immunogenic endotoxin, lipopolysaccharide (LPS), from *P. gingivalis* [15].

Focal dissemination of periodontal pathogens from the oral cavity to distant organ sites has long been hypothesized, but few studies have explored this theory. Previous studies using wild-type mice (C57BL/6J) explored the dissemination of periodontal pathogens in an endodontic infection model [36]. However, the study detailed here was unable to trace the dissemination of periodontal pathogens to distant organ sites due to the disadvantages associated with using a wild-type mouse model [36]. The ApoE$^{-/-}$ mouse model, which is a proatherogenic model for co-morbidity studies, is unable to deposit Aβ in the brain as the essential ApoE isoforms are lacking [37]. This mouse serves

as a suitable model with which to study the association between periodontal disease and AD as it avoids confounding factors that may result from an overlap of signaling in response to AD hallmark proteins and pathogen-associated molecular patterns. Thus, keeping in view the lack of *in vivo* experimental evidence for a link between periodontal pathogens/disease and AD, the present study aimed to explore such an association using the ApoE$^{-/-}$ mouse as a model. This study also tested the hypothesis that infectious agents and/or their components from oral diseases such as periodontitis can access the brain and modulate local CNS inflammation. To this end, we investigated the role of the oral pathogens *P. gingivalis*, *T. denticola,* and *T. forsythia* in accessing the brain of ApoE$^{-/-}$ mice following chronic experimental periodontitis and in contributing to the development of local inflammation as an early pathological lesion in relation to AD.

The present study explored the possibility of specific oral pathogens altering normal functioning of the brain in experimental animals with established periodontitis. In this infection model *F. nucleatum* was used as a bridging organism that co-aggregates with major periodontal bacteria in both supra- and subgingival biofilm development and for the subsequent progression of periodontitis [38–40].

MATERIALS AND METHODS

Mice, oral infection, and brain

The study involved oral infection of ApoE$^{-/-}$ mice with periodontal pathogens either as mono- or polybacterial for a chronic infection period of 24 weeks. Following the infection period the mice were euthanized and the brain tissue was collected and preserved. Later, using molecular, immunological, and pathological detection techniques we evaluated the invasion of periodontal bacteria into the mice brains.

Microbial strains

P. gingivalis FDC 381, *T. denticola* ATCC 35404, *T. forsythia* ATCC 43037, and *F. nucleatum* ATCC 49256 were used in the study and were routinely cultured anaerobically at 37°C as described previously [41].

ApoE$^{-/-}$ mice oral infection

Eight-week-old male ApoE$^{-/-}$ mice strain B6.129P2-Apoe$^{tm1Unc/J}$ (Jackson Laboratories, Bar Harbor, ME, USA) were randomly assigned to sham-infected, mono-infected (*P. gingivalis, T. denticola, T. forsythia,F. nucleatum*) and polymicrobial-infected groups ($n = 12$ in each group). This mouse study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, USA. All procedures were performed in accordance with the approved protocol guidelines (Protocol # 201004367) set forth by the Institutional Animal Care and Use Committee of the University of Florida. The University of Florida has an Assurance with the Office of Laboratory Animal Welfare and follows Public Health Service policy, the Animal Welfare Act and Animal Welfare Regulations, and the *Guide for the Care and Use of Laboratory Animals*, USA. ApoE$^{-/-}$ mice were administered with 500 μg/mL kanamycin in drinking water for 3 days followed by a mouth rinse with 0.12% chlorhexidine gluconate [42] before the first oral lavage with the periodontal bacteria [42] to suppress the murine indigenous oral microflora. While mono-infections involved a bacterial inoculum of 10^9 cells/mL of respective bacteria, the polymicrobial-infection constituted an inoculum of 5×10^9 combined bacteria/mL, as described previously [41, 42]. This investigation is part of an on-going collaboration with the University of Florida and the University of Central Lancashire (UCLan) (MTA Ref. No. A10415). Ethical approval was obtained from the Animal Projects Committee of UCLan for research on animal tissues as secondary users (Ref. No. RE/11/01/SS).

Collection and storage of brain tissue specimens

The mouse brains were removed following 12 and 24 weeks of oral infection as well as sham-infection and separated into two halves. One cerebral hemisphere was immediately stored at −80°C in RNA*later*® buffer for subsequent molecular biology analysis and the other half fixed in 10% neutral buffered formalin for histopathological analysis.

Genomic DNA Isolation

To confirm the spread of periodontal pathogens from the mouth to the brain of ApoE$^{-/-}$ male mice, genomic DNA was isolated from the brains of all the infected and sham-infected groups. Briefly, frozen brain tissue (25 mg) was removed, close to the circumventricular organs in a bench top microflow cabinet (Astec Microflow Ltd., UK), using the aseptic technique [15].

Table 1a
PCR primers from Paster et al. [43]

Primer	Function	Orientation	Sequence
D88	PCR	Forward	GAGAGTTTGATYMTGGCTCAG
E94	PCR	Reverse	GAAGGAGGTGWTCCARCCGCA

Table 1b
Specific primer sets used for analysis of bacterial DNA from ApoE$^{-/-}$ mice brains by PCR

Primer [Ref]	Amplicon size	Primer	Sequence
P. gingivalis [44]	PCR	Forward	AGGCAGCTTGCCATACTGCG
P. gingivalis [44]	PCR	Reverse	ACTGTTAGCAACTACCGATGT
T. denticola [41]	PCR	Forward	TAATACCGAATGTGCTCATTTACAT
T. denticola [41]	PCR	Reverse	CTGCCATATCTCTATGTCATTGCTCTT
T. forsythia [44]	PCR	Forward	GCGTATGTAACCTGCCCGCA
T. forsythia [44]	PCR	Reverse	TGCTTCAGTGTCAGTTATACCT
M13 (Invitrogen)	Sequencing	Reverse	CAGGAAACAGCTATGAC

Following the manufacturer's protocol (Qiagen DNA easy blood & tissue kit 69504), brain tissue was lysed and genomic DNA was isolated manually using ethanol precipitation.

DNA amplification and sequencing

Polymerase chain reaction (PCR) was performed using a thermocycler (Veriti, Applied Biosystems, UK), initially using the universal bacterial primers (Table 1a) from the 16 s rDNA bacterial genes [43]. For the bacterial-specific gene amplification, the primer sets from Figuero et al. [44] and Rivera et al. [41] (Table 1b) were employed, adhering to the published PCR protocols [41, 44]. PCR products were analyzed using agarose gel electrophoresis (1.5%) and visualized in the Gene Genius bio-imaging system, and images were captured using the Gene snap software (Syngene, UK). The PCR product was cleaned in MicroCLEAN DNA Cleanup® reagent (Web Scientific Ltd.) and cloned using the TA TOPO cloning kit (Invitrogen) according to the manufacturer's instructions. Following successful colony screening, a mini culture (10 ml) of each of the selected colonies was set up overnight and plasmid DNA isolated using a Qiaquick kit (Qiagen). This was followed by sequencing (40 ng) with the M13 forward or reverse primers (TA TOPO cloning kit, Invitrogen) and using the BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The sequencing parameters were an initial denaturation step at 96°C for 1 min and 25 cycles involving (96°C for 10 s), annealing (50°C for 5 s), and elongation (60°C for 4 min) according to Paster et al. [43]. Following sequencing the results were submitted to BLAST nucleotide search engine for 16 s DNA genes (http://blast.ncbi.nlm.nih.gov/) to identify the organism(s) with 99–100% match with at least 200 bases.

Immunodetection of periodontal pathogens in mouse brain tissue

Isolation of total protein from mouse brain tissue

In each case a 3-mm-thick section of the cortical brain was minced in the lysis buffer containing protease inhibitors [15]. The total protein concentration of all cell lysates was determined as described previously [15]. A number of positive and negative controls were kindly provided as gift reagents and their sources are identified in Table 2. These were sterile bacterial growth medium (medium control) and *P. gingivalis* culture supernatant as described in Poole et al. [15], purified recombinant *T. denticola* protein (FhbB) [45], and ready-to-use *T. forsythia* whole-cell lysate [46].

Immunoblot analysis

Immunoblotting was performed under reducing conditions in which up to 60 μg per lane of total protein from all brain specimens was loaded [15] on SDS-PAGE gels of variable percentages (7.5% gels were used for high-molecular-weight proteins such as the S-layer of *T. forsythia*, 12.5% for gingipains and LPS from *P. gingivalis* and 15% w/v gels were used for the low-molecular-weight proteins detected by anti-*T. denticola* antibodies). Following electrophoresis, proteins were electro-transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore, UK). The membranes were blotted with mouse anti-*P. gingivalis* (clone 1B5), rabbit anti-*T. forsythia* against the S-layer, and anti-*T. denticola* ATCC 35405 antibody against

Table 2
Source of antibodies and their working concentration and/or dilutions used

Antibody	Supplier	Final conc/ dilution
Rabbit anti-GFAP (gift)	Dr Jia Newcombe (The Multiple Sclerosis Society Laboratory, UK)	1/1000
Goat anti-Iba 1 (ab5076)	Abcam	1/250
Mouse anti-*P. gingivalis* (Clones 1B5) tissue culture supernatant (gift)	Prof. Michael A. Curtis (London, UK)	1B5 1/10
Rabbit anti-*T. forsythia* (S-layer protein)	Dr Graham Stafford (University of Sheffield, UK)	1/20,000
Rat anti-*T. denticola* (FhbB protein)	Prof. Thomas T. Marconi (USA)	1/5,000
Blocking solution	0.01 M phosphate buffered saline, pH 7.3, containing 0.01% normal goat or rabbit serum and 0.25% tween 20	–
Normal serum: goat (X0907), rabbit (X0902).	DakoCytomation (Germany)	0.01%
Rat anti-mouse C3b/iC3b/C3d	Hycult Biotechnology (UK)	1/50
Rabbit anti-rat C9 neoepitope	Professor B. Paul Morgan, and Dr Timothy R. Hughes (Cardiff University)	1/100

FhbB protein generated in rats (sources of antibodies and their dilutions used are listed in Table 2).

Histopathological staining of brain tissue

The formalin-fixed brain tissue was thoroughly washed in PBS and the intact hemisphere was divided into the frontal cortex, temporal lobe inclusive of the hippocampus, and the brain stem and cerebellum. The specimens were then processed and embedded in paraffin wax. The tissue blocks with temporal lobe inclusive of the hippocampus were sectioned (5 μm in thickness) using the Leica RM2235 microtome.

Cryo-sections (10 μm thickness) from frozen unfixed brain tissue (hippocampus) were cut using the Leica CM1850 cryostat (Leica UK). Both paraffin wax and cryo-sections were collected onto superfrost+® glass slides (Leica UK). The cryo-sections were either used immediately or stored at −80°C until required for further use. Rehydrated paraffin wax sections were examined for morphology following staining with Hematoxylin and Eosin (H&E). In addition, a modified methenamine silver (silver impregnation) technique adapted from resin-embedded-tissue specimens as previously described by Singhrao et al. [47] was used to demonstrate the Aβ plaques and the NFTs. All sections were also stained with 1% aqueous thioflavin T as a standard neuropathology technique for detecting fibrillar amyloid deposition.

Immunofluorescence labeling of periodontal pathogens in brain tissue

Antigen retrieval was carried out on rehydrated paraffin wax sections for labeling with goat anti-Iba1 (Abcam) by microwave heating of tissue sections at 750 W power for 35 min in 10-mM citric acid buffer (pH 6.0). The infected as well as sham-infected control brain sections were incubated in primary antibodies and subsequently in secondary detection antibodies. Rehydrated paraffin wax sections were immunolabeled with rabbit anti-glial fibrillary acidic protein (GFAP) (Table 2) and the calcium binding protein marker Iba 1 (AbCam). For formalin fixative sensitive antibodies, tissue sections from frozen brains were stabilized by fixation in cold acetone for 10 min followed by a 5-min wash in PBS. Tissue-associated endogenous fluorescence was quenched for 10 min in 50-mM glycine/PBS. All brain tissue specimens were immunolabeled using the mouse anti-*P. gingivalis* (1B5), anti-*T. denticola* against FhbB protein, and anti-*T. forsythia* (against S-layer) and for complement C3 activation products rat anti-C3b/iC3b/C3d (Hycult Biotech), and a rabbit anti-C9 neoepitope to detect the membrane attack complex. The dilutions for incubation of sections in primary antibodies are given in Table 2. Where appropriate, the antibodies were diluted in block solution containing 0.01% normal serum (goat serum for GFAP, *P. gingivalis* (1B5), *T. denticola* (FhbB), *T. forsythia* (S-layer), C3b/iC3b/C3d and C9 neoepitope; rabbit serum for Iba 1) in PBS pH 7.3 and 0.25% tween 20. FITC-conjugated secondary detection antibodies were goat anti-rabbit (Sigma-Aldrich Ltd., UK) diluted 1/200 and rabbit anti-goat Alexa Fluor 488® and goat anti-rat Alexa Fluor® 488 (Molecular Probes, UK) diluted 1/1000, in block solution. Sections were mounted under a glass coverslip using the Vectashield® PI (propidium iodide) mounting medium (Vector laboratories, Perterborough, UK). Labeling was observed and images were captured using a 510 series Zeiss confocal microscope (Carl Zeiss Ltd). A semi-quantitative approach was taken by manually counting the number of cells/area for all

Table 3
DNA detected from periodontal pathogens in the ApoE$^{-/-}$ mice brains

Mono infections	DNA detected at 12 weeks	DNA detected at 24 weeks	Polymicrobial infections 12 weeks	Polymicrobial infections 24 weeks
Sham-infected	0 out of 12	0 out of 11	0 out of 11	0 out of 11
P. gingivalis	6 out of 12, $p = 0.006$	9 out of 11 $p = 0.0001$	0 out of 11	2 out of 11
T. denticola	0 out of 12	0 out of 12	0 out of 11	0 out of 11
T. forsythia	0 out of 12	0 out of 12	0 out of 11	0 out of 11

brains in each infected group and compared with the sham group to assess glial cell activation.

Statistical analysis

Data are presented as mean ± standard deviation ($n \geq 3$ replicates per treatment) and tested for normality and equal variance prior to analysis. Where treatment groups did not meet the assumptions for parametric analysis, the non-parametric Mann Whitney-U test was performed comparing the number of positive cases in each group of infected mice with those in the sham-infected group. Differences were considered significant at $p \leq 0.05$.

RESULTS

Molecular identification of pathogens in brain specimens

Molecular analysis using universal primers failed to detect *T. denticola* or *T. forsythia* in the brain tissues from sham-, mono-, and polymicrobial-infected groups at both time intervals (Fig. 1a-c). The species-specific bacterial gene primers revealed 6 out of 12 ApoE$^{-/-}$ mice brain specimens containing *P. gingivalis* genomic DNA at 12 weeks (Fig. 1d), which further increased to 9 out of 12 at 24 weeks (Fig. 1e). These results are highly significant when analyzed by the non-parametric Mann Whitney-U test; $p = 0.006$ at 12 weeks and $p = 0.0001$ at 24 weeks. The molecular identity of the organism was further confirmed following purification of the amplification product and direct sequencing. A nucleotide basic local alignment search tool (BLAST) identified a 99-100% match with >200 bases of the submitted sequence for *P. gingivalis*. Following molecular identification using specific bacterial gene primers, the group of brains from the polymicrobial infections failed to detect *P. gingivalis* genomic DNA at 12 weeks. However, by 24 weeks 2 out of 12 ApoE$^{-/-}$ mice brain specimens demonstrated the presence of *P. gingivalis* genomic DNA (Fig. 1f). The brain tissue sections from polymicrobial-infected

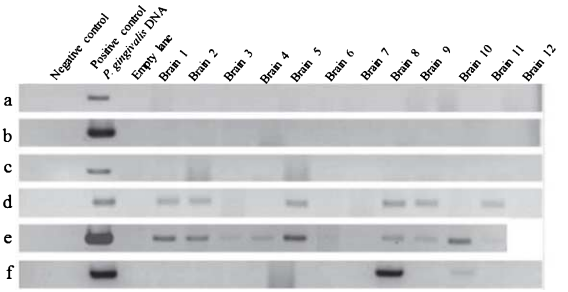

Fig. 1. Molecular identification of *P. gingivalis* in brain tissue sections using specific primers. Panels a and b) mono sham-infected group 12 and 24 weeks, c) polymicrobial sham-infected group 24 weeks, d) Mono- infection with *P. gingivalis* at 12 weeks, e) Mono-infection with *P. gingivalis* at 24 weeks, f) Polymicrobial infection with *P. gingivalis* at 24 weeks. d) Lanes corresponding to Brain 1, 2, 5, 8, 9, 11 demonstrated a band at 400 bp. $p = 0.006$. e) Lanes corresponding to Brain 1, 2, 3, 4, 5, 6, 8, 9, 10, 11 demonstrated a band at 400bp. $p = 0.0001$. f) Lanes corresponding to Brain 8 and 10 demonstrated a band at 400 bp.

mice did not show the presence of *T. denticola* and *T. forsythia* at either 12 weeks or 24 weeks (Table 3).

Immunoblot analysis of infected mouse brain tissue

None of the test tissue lysates demonstrated LPS, FhbB protein, and the S-layer protein from their respective bacterial species in the mono- and polymicrobial-infected groups (data not shown).

Histology of the infected mouse brain

Overall morphological observations of the temporal lobe, including the hippocampus, appeared well preserved in H&E preparations obtained from all brains (Fig. 2). The pyramidal neurons in all sub-regions of the hippocampus (CA1-CA4) and the dentate gyrus in sham-infected and infected brains generally also appeared to be well preserved (Fig. 2a-d). Occasionally, shrunken and darker neurons were noted to a varying extent in CA1-CA4 regions and the dentate hilus with a random distribution (not shown). There were no abscesses in the brain and there were no signs

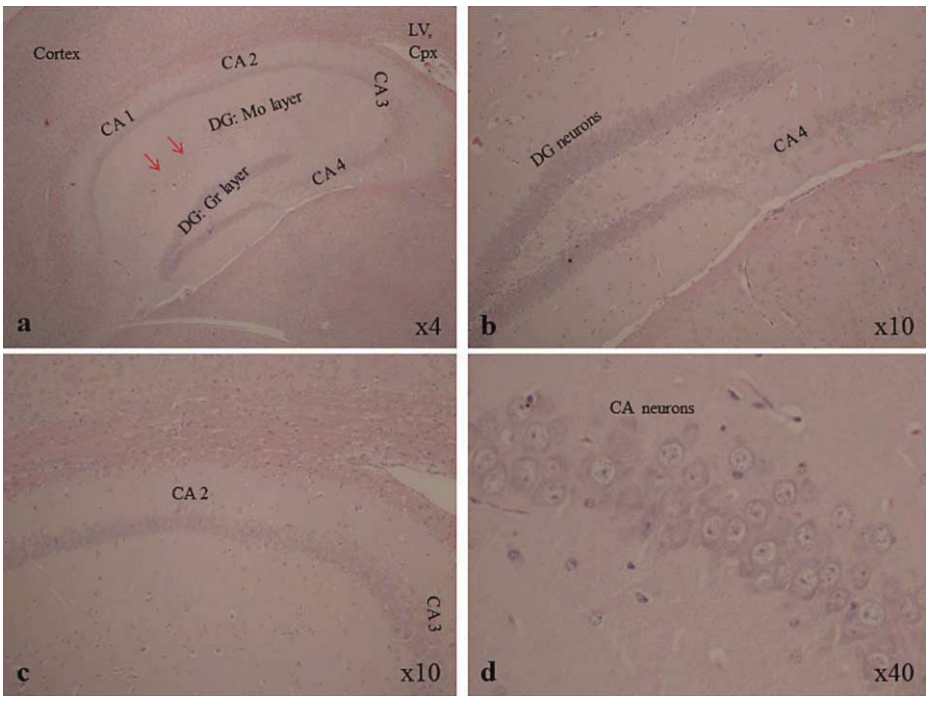

Fig. 2. Hematoxylin and Eosin stained tissue section from the temporal lobe of ApoE$^{-/-}$ mice demonstrating the overall preservation of a) CA1-CA4 regions of the hippocampus, b) Higher magnification of the dentate gyrus neurons, c) the cortical and hippocampal fissure by the lateral ventricle in relation to CA2 and 3 neurons, d) higher magnification of the CA2 neurons. DG: Gr layer, dentate gyrus granule cell layer. The red arrows depict fused hippocampal fissure. LV, lateral ventricle containing the choroid plexus.

of the classical blood-borne inflammatory cells (neutrophils, lymphocytes) or sites of focal hemorrhage. Thioflavin T and methenamine silver neutral staining methods failed to demonstrate any evidence for the presence of either Aβ plaques or NFTs in the hippocampus or in the frontotemporal cortex regions in all of the brains examined.

Immunofluorescence detection of periodontal pathogens in infected mouse brain tissue

Cell markers associated with glial cell activation

Astrocytes (GFAP): All the sections from the sham-infected brains and mono- and polymicrobial-infected groups in which the primary antibody was omitted remained negative (Fig. 3a, d).

Immunolabeling of sections for GFAP in the sham-infected control brains demonstrated numerous astrocytes with activated phenotypes around the lateral ventricles (Fig. 3b) as well as scattered astrocytes within the hippocampus CA1-CA4 regions at both time points (Fig. 3c). The brain tissue sections from *P. gingivalis* mono-bacterial-infected groups at 12 and 24 weeks showed astrocytes at the periphery of the lateral ventricles (Fig. 3e) and within the hippocampus (Fig. 3f). There was no statistical difference when cells/area were counted and compared with the sham-infected mice. The brain tissue sections from *T. denticola* mono-infected groups at 12 and 24 weeks demonstrated a similar density of astrocytes scattered at the periphery of the lateral ventricles and within the hippocampus (not shown) as observed in the *P. gingivalis*-infected and sham-infected mice. The brain tissue sections from *T. forsythia* mono-infected groups at 12 and 24 weeks demonstrated a lower density of astrocytes scattered at the periphery of the lateral ventricles and within the hippocampus compared with the *P. gingivalis* and *T. denticola* groups as well as the sham-infected mice (not shown). Equally, the polymicrobial-infections demonstrated no significant difference compared with the control group. GFAP labeling was observed in the circumventricular regions as well as in the hippocampus (not shown).

Microglia (Iba 1): All mouse brain sections in which the primary antibody was omitted remained negative for microglial cell distribution (Fig. 4a, d). Only a few microglial cells were observed following immunolabeling of sections with the Iba 1 antibody around the

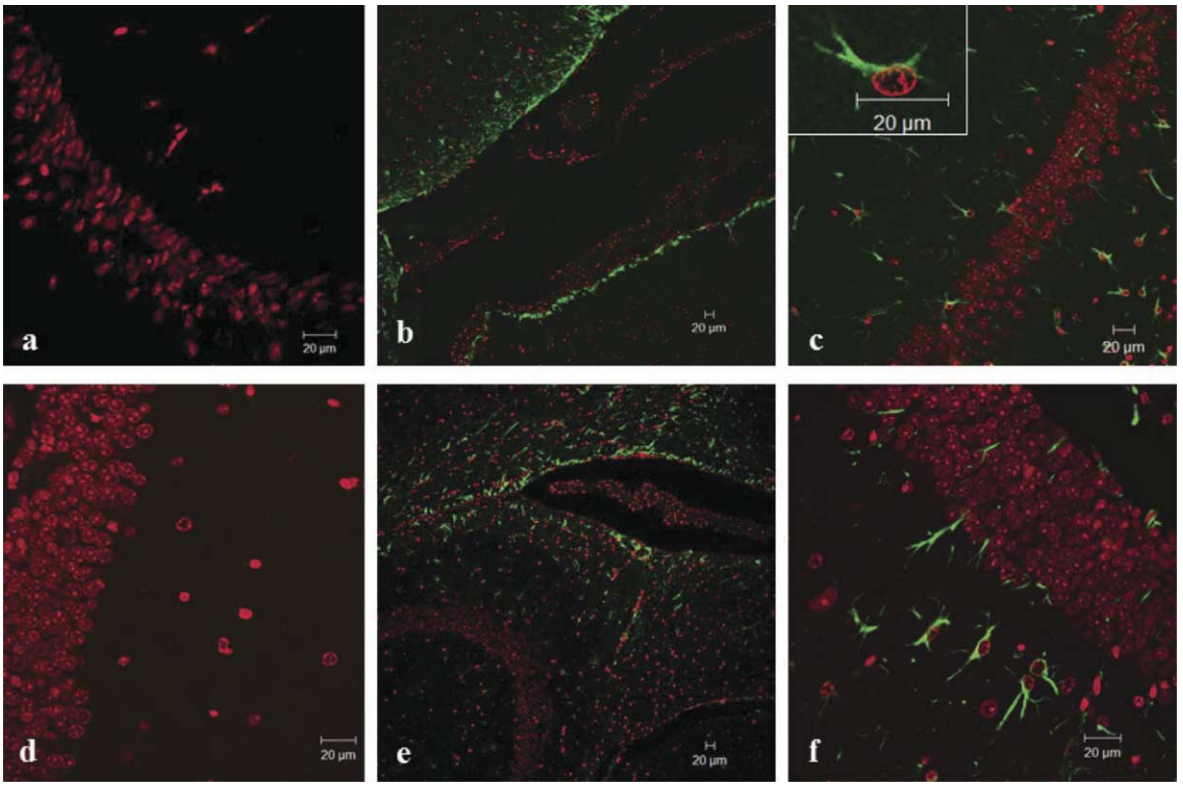

Fig. 3. Immunolabeling of the temporal lobe of ApoE$^{-/-}$ mice with rabbit anti-human GFAP to assess astrogliosis. a and d) negative control images whereby primary antibody is omitted. Sham-infected (b, c) in which (b) demonstrated abundance of immunopositivity especially around the periphery of the lateral ventricles and the inset in (c) shows the morphology of cells labeled with anti-GFAP. These appeared as fibrillary astrocytes with reactive phenotype. The mono *P. gingivalis* infected (e, f) brains at 24 weeks demonstrated a more widespread distribution of fibrillary astrocytes around ventricles but their distribution within the hippocampus region was similar to that observed in the sham-infected brains.

lateral ventricles at 12 and 24 weeks in the sham-infected brain sections (Fig. 4b), with even fewer cells (mainly processes, Fig. 4c) in the hippocampus. Similar microglial cell distribution was observed in the *P. gingivalis*-infected brains around the lateral ventricles (Fig. 4e), and few microglial cell bodies with branched processes were observed in the hippocampus (Fig. 4f). The brain tissue sections from *T. denticola* mono-infected groups at 12 and 24 weeks demonstrated no differences in the density of microglia scattered around the periphery of the lateral ventricles or within the hippocampus (not shown). Similarly, there were no differences observed between sham-infected, *T. forsythia*-infected, and polymicrobial-infected brain sections.

Detection of bacterial virulence factors in infected mouse brain tissue

Immunolabeling of brain cryo-sections was unable to demonstrate the presence of any of the three bacteria used for infection when tested using anti-*P. gingivalis* antibody, rabbit antisera against *T. forsythia*, and anti-*T. denticola*.

Detection of complement activation proteins in mouse brain tissue

The sham-infected mouse brain sections, in which the primary antibody was omitted, remained negative for C3 complement activation products (Figs. 5a, 6a). Intracellular labeling detected complement activation products for the common C3 component activation fragments (iC3b, C3b and C3d) (Figs. 5b, 6b) and the membrane attack complex C9 neoepitope (Fig. 6c), specifically on microglia rather than on astrocytes and/or neurons from all brain tissues in sham-infected mice. The complement activation products for the common C3 components (iC3b, C3b, and C3d) and C9 (C9 neoepitope) were detected in *P. gingivalis*-infected mouse brains (12 weeks), but the labeling was intracellular and exclusive to microglia. By 24 weeks, the glial cell labeling was still high (Fig. 5c), but C3 (Fig. 6d, e),

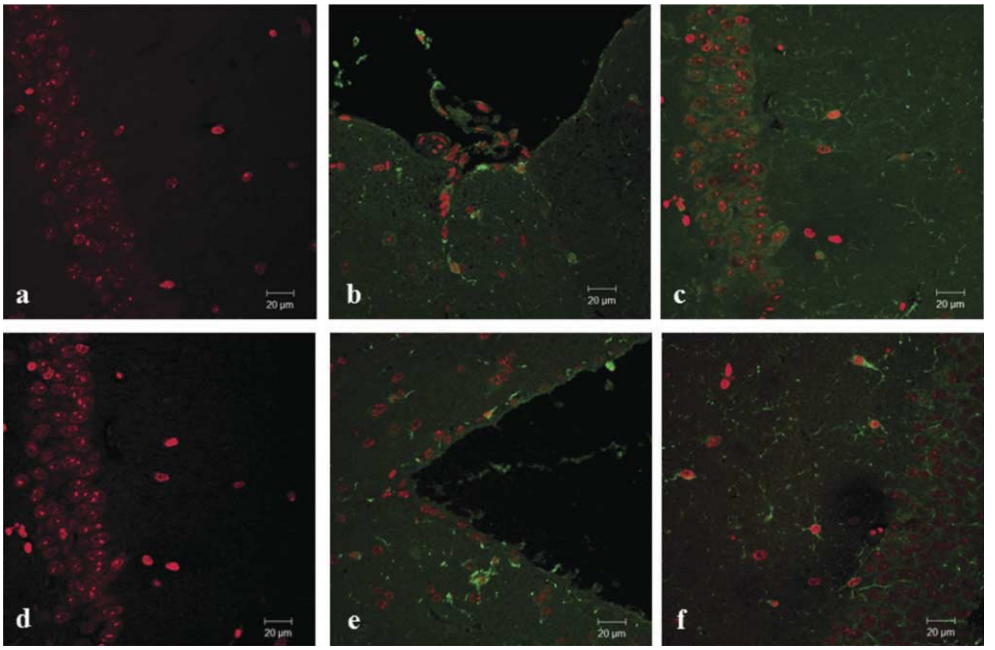

Fig. 4. Immunolabellng of the temporal lobe of ApoE$^{-/-}$ mice with goat anti-mouse Iba1 antibody to assess microgliosis. a and d) negative control images whereby primary antibody is omitted. Sham-infected (b, c) in which (b) demonstrated immunopositivity around the periphery of the lateral ventricles. The mono- *P. gingivalis* 24 weeks infected (e, f) brains demonstrated similar labeling to that observed in the sham-infected brains, in both the lateral ventricles and hippocampal regions.

and C9 (Fig. 6f) activation fragments appeared to be opsonized onto pyramidal neurons, particularly in the CA2 area of the hippocampus in 4 out of 12 infected brains ($p = 0.032$). Labeling of the C9 neoepitope was observed in 2 out of 12 specimens ($p > 0.05$, Fig. 6f). In contrast, both *T. denticola* and *T. forsythia* infections (12 weeks) were similar to the control mice, demonstrating intracellular staining in microglial cells. However, at 24 weeks, 1 out of 12 from each group demonstrated both C3 (iC3b, C3b, and C3d) and C9 neoepitope localized to CA neurons ($p > 0.05$) (data not shown). Immunolabeling of polymicrobial-infected mouse brains (12 and 24 weeks) with the same antibodies also demonstrated the glial cells.

DISCUSSION

Infectious agents have previously been linked to cognitive decline [9–13], and more recently periodontal pathogens and/or their virulence factors have been implicated in the development of AD [14, 15]. This study explored the hypothesis that infectious agents and/or their components from oral diseases such as periodontitis can access the brain and contribute to local CNS inflammation that eventually leads to the development of a chronic inflammatory component of AD. In this study we investigated the possibility that oral pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* can access the brains of ApoE$^{-/-}$ mice following experimental induction of periodontitis as mono- as well as polymicrobial-infections. *F. nucleatum* has the ability to co-aggregate with early colonizers in the oral cavity as well as the late colonizers such as *P. gingivalis*, *T. denticola*, and *T. forsythia* [36–38]. However, in the present study no attempt was made to detect *F. nucleatum* in the brain specimens as *F. nucleatum* is part of another ongoing study. The significance of using a periodontal disease model to assess AD lies in understanding the role of bacteria accessing the brain and thereby priming glial cells to mount a subsequent local immune response and contribute to neuronal lysis. One previous study, which was performed with an endodontic infection model using wild-type and the severe-combined-immunodeficiency (SCID) mice, demonstrated that only the SCID mice were conducive to *T. denticola* invasion following mono- and polymicrobial-infections [36]. That study showed that *T. denticola* can disseminate to distant body organs, including the brain, heart, and spleen while *P. gingivalis* and *T. forsythia* were undetected [36]. In our current study using a periodontal infection model in ApoE$^{-/-}$ mice, we report a contrasting

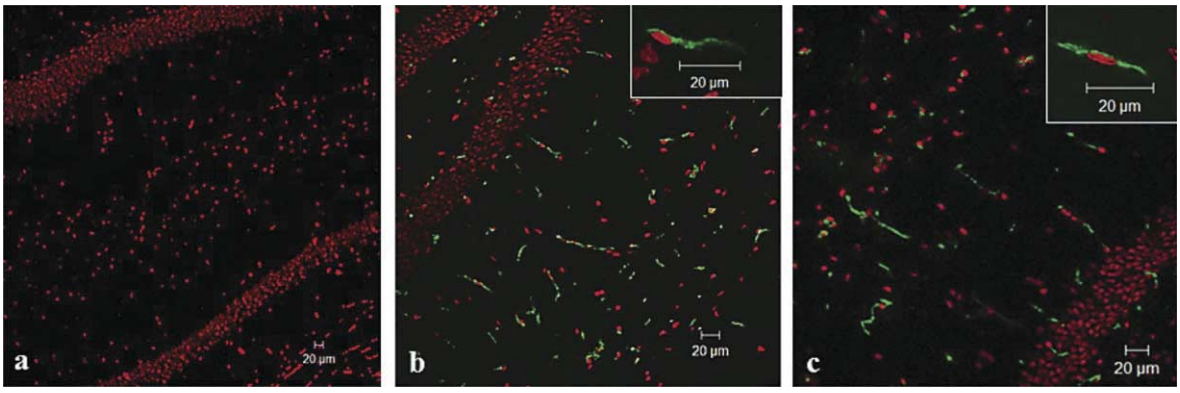

Fig. 5. Cryo-section from the temporal lobe of ApoE$^{-/-}$ mice immunolabeled for complement activation fragments in the hippocampus using rat anti-mouse C3b/iC3b/C3d. (a) Control, where the primary antibody was omitted from the tissue section. In both sham-infected (b) and infected (c) brains, the labeling appears intracellular within branched microglia demonstrating an activated phenotype. The inset (b-c) shows the branched morphology of cells labeled with the same antibody.

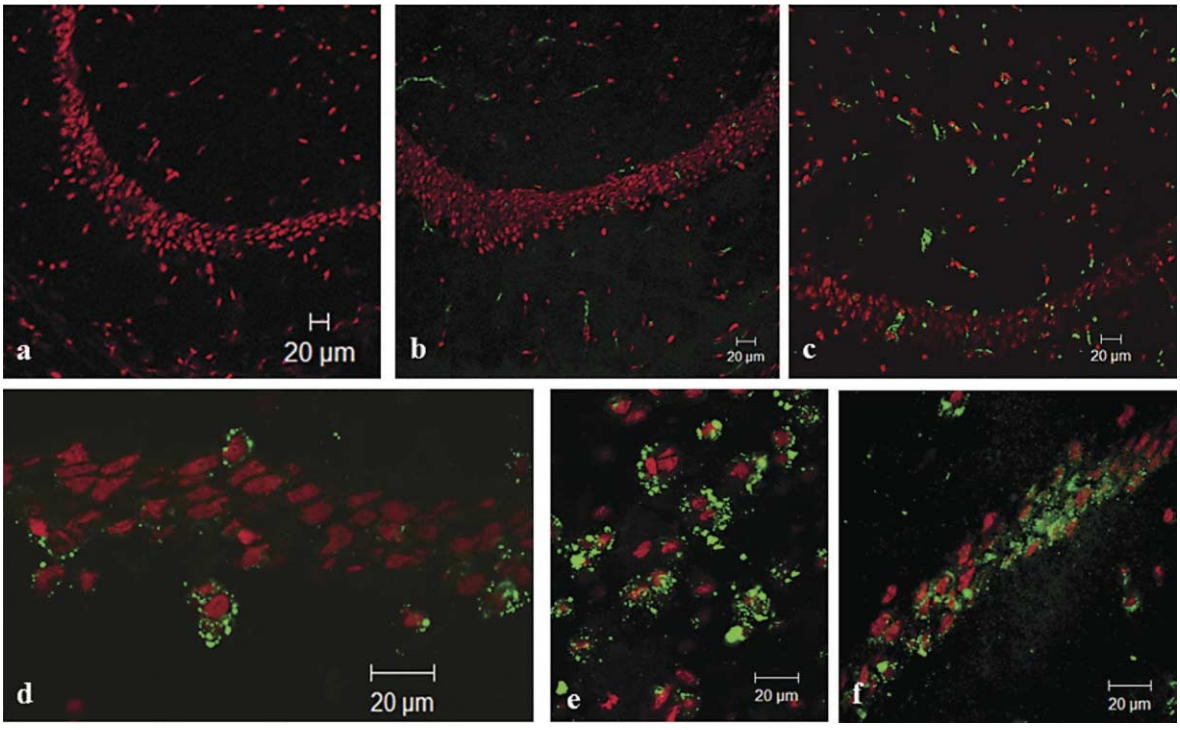

Fig. 6. Immunodetection of complement fragments in brain tissue sections using rat anti-mouse C3b/iC3b/C3d. (a) Negative control (b–c) sham-infected brains with rat anti-mouse C3b/iC3b/C3d (b) and rabbit anti-rat C9 neoepitope (c). (d–f) *P. gingivalis* infected brain with rat anti-mouse C3b/iC3b/C3d (d and e) and rabbit anti-rat C9 neoepitope (f); showing labeling on the cell surface membranes of the CA neurons in the infected brains ($p = 0.032$).

finding in which we observed the dominance of *P. gingivalis* in accessing the brain in comparison to *T. denticola* and *T. forsythia*. These differences in our study from those of Foschi et al. [36] maybe due to the bacterial strains used, the dosage of infection administered, method of inoculating animals during infection, differences in disease models (endodontic versus periodontal disease), as well as the genetic makeup of the mice used. For example, the only common strain between this study and that of Foschi et al. [36] is *T. forsythia* (ATCC 43037) and the dose of bacteria used in each study was different (higher by a factor

of 10 in this study). Based on the available data it is likely that *T. forsythia*, being a non-motile bacterium which lacks fimbriae, is unable to transmigrate to the brain [48]. We found that *P. gingivalis* FDC381 DNA predominated in the brains of ApoE$^{-/-}$ mice, and this strain is highly fimbriated compared to the *P. gingivalis* ATCC 33277 [48] used by Foschi et al. [36]. Although both strains of *T. denticola* are motile, the *T. denticola* (ATCC 35405) used by Foschi et al. [36] at a lower dose disseminated to the brain. This difference may be attributed to the outer membrane, with abundant pore-forming adhesion protein that may be lacking in our *T. denticola* (ATCC 35404) strain [49]. Thus, the virulence of the bacteria may have contributed to its accessibility to the brain, rather than being a dose-dependent effect.

Despite the differences in bacterial strains used and their dosage, as well as the genetics of the experimental animals, our results show that *P. gingivalis* strain FDC 381 used to infect the oral cavity of the ApoE$^{-/-}$ mice was able to access the brain tissue, providing definitive evidence for transmigration of this bacterial species from the oral cavity to the brain. The fact that more brains demonstrated a greater *P. gingivalis* infection at 24 weeks of infection suggests that the translocation of bacteria is likely to be time dependent. Inflammation occurring at 24 weeks of infection may be increasing the permeability of the blood-brain barrier and facilitating easier access of bacteria into the brain.

Detecting *P. gingivalis* in the ApoE$^{-/-}$ mice brains in this *in vivo* study supports the data presented in our recently published study of human brain specimens in which we detected *P. gingivalis*-specific LPS in 4 out of 10 AD human brains [15]. Together these studies provide evidence to support an association between periodontal disease and AD. When examined for general morphological preservation of the frontotemporal lobe, including the hippocampus, rehydrated paraffin wax sections showed no signs of abscess formation, no myeloid lineage cells (neutrophils, lymphocytes) infiltrating into the brain, and no sites of focal brain hemorrhage.

Our immunoblotting and immunofluorescence techniques with specific antibodies did not show the presence of bacterial virulence factors in any of the brain tissues examined. If any of these are metabolically active in the brain, it may take several years to form an abscess as seen in the case with non-oral bacteria such as *Propionibacterium acnes*, which can take 10 years to form an abscess following entry into the brain [50]. Although this appeared surprising at first, the lack of detection may be attributed to the inability of these bacteria to access the brain due to their rapid clearance from the systemic circulation and/or they were neutralized upon entry by the already enhanced microglial cell inflammatory phenotype in these mice [51, 52]. Another possible reason may be that the antibodies themselves failed to detect their epitope in tissue sections or the antigen itself was below the detection limit of both immunoblotting and immunolabeling.

We focused on the hippocampus region of the brain to detect any early cellular changes in the ApoE$^{-/-}$ mice brains, as according to Braak and Braak [53] neurodegeneration begins in the entorhinal cortex and spreads to the hippocampus followed by other regions. Screening for the AD hallmark associated structures by thioflavin T and methenamine silver methods failed to provide any evidence for the fibrillar Aβ and NFTs in the entorhinal cortex or the hippocampus regions. A plausible reason for the inability to detect the AD hallmark proteins could be the relatively short time span of chronic infection in our mouse model because, even in the accelerated transgenic AD animal model and in the AβPP and SS-1 transgenic mice, insoluble Aβ deposition and plaque formation usually takes between 6 to 12 months [54, 55]. Further, ApoE$^{-/-}$ mice used in the current study are unlikely to demonstrate Aβ deposition as they lack the essential protein required for amyloid to form insoluble fibrils [37]. Hence it will be beneficial for a future study to be designed with a longer duration of mono- and polymicrobial-infection in a non-ApoE$^{-/-}$ rodent model so as to demonstrate the direct link between periodontal disease and AD hallmark proteins.

Previous studies with ApoE$^{-/-}$ mice have identified glial cell activation in which microglia demonstrate evidence of an increased secretion of cytokines, especially of tumor necrosis factor-α (TNF-α) [51, 52], a cytokine of macrophage origin. This observation has been suggested as an impaired immuno-modulatory function of macrophages in controlling the innate immune responses in this animal model [56–58]. Microglial cells are the tissue-bound macrophages of the brain capable of expressing a range of proinflammatory cytokines and phagocytosing cellular debris to reduce the inflammatory response to pathogens. However, the finding that the ApoE$^{-/-}$ mice have higher levels of endogenous proinflammatory cytokines, especially TNF-α suggests that it is likely that microglia were already in their primed phenotype. In this study we also found responsive fibrillary astrocytes, particularly at the peri-circumventricular organ sites following initial microglial cell activation. Complement is a pivotal pathway in the CNS innate

immune response following infections. In the CNS, the dominant mode of complement activation is the classical pathway where neurons show vulnerability to complement mediated damage [59] and microglia synthesize complement proteins [60]. Hence, we set out to detect any evidence for the activation of the common C3 and the terminal pathway of complement leading to the formation of the membrane attack complex in our infected mice brain specimens. Our study demonstrated an intracellular localization of C3 and C9 exclusively in microglia in all brains, suggesting that these cells were actively synthesizing complement components [60] rather than being opsonized with the complement activation fragments, again supporting the view that microglia were already in their primed/activated state [51, 52, 61].

However, our observation of the cell surface membrane staining of C3 activation fragments (iC3b, C3b, and C3d) and the membrane attack complex (anti-C9 neoepitope) exclusively on CA pyramidal neurons of the mono- and polymicrobial-infected mice at 24 weeks but not at 12 weeks suggests that the inflammatory burden was increasing from protection to causing bystander injury on complement activated neurons. In view of us detecting C3 activation fragments being opsonized on the pyramidal neurons, it appears likely that bacteria (*P. gingivalis*) and/or its DNA may have triggered the complement activation in these infected mice.

Our study supports the observation from previous studies which hypothesized that bacterial infections would contribute to the development of AD pathology via mechanisms involving acute-phase proteins, including cytokines and the complement cascade in which neurons would be attacked [31–33]. The presence of cytokines and activated complement cascade can be used as a marker to represent local CNS inflammation [1, 33]. Thus, the demonstration of activated complement cascade here in response to *P. gingivalis* directly infecting the brain supports the conclusion that chronic local inflammation constitutes a component of developing AD pathology.

Finally, this study demonstrates that, in the absence of fibrillary Aβ deposition the neurons remain vulnerable to complement mediated damage from *P. gingivalis* accessing the brain.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2354).

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Bacterial Burden in Disease, Aging and Alzheimer's Disease

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Abstract. This review shows how our microbiome influences health and ultimately how well we age. Evidence linking oral bacteria to Alzheimer's disease (AD) is discussed in the context of aging, drawing together data from epidemiological, experimental, genetic and environmental studies. Immunosenescence results in increased bacterial load as cell-mediated and humoral immune responses wane, with the innate immune system contributing to a rise in circulating proinflammatory cytokines such as TNFα and IL1β. Aging may favor the proliferation of anaerobes in the mouth eliciting a robust TNFα response from the oral epithelium. Maintaining the integrity of the blood-brain-barrier (BBB) against a backdrop of increasing bacterial load is important; prolonged exposure to high levels of TNFα compromises its integrity. Sensitive techniques now detect the "asymptomatic" presence of bacteria in areas previously thought as sterile, providing new insights into the wider distribution of components of the microbiome. These "immune-tolerated" bacteria may slowly multiply elsewhere until they elicit a chronic inflammatory response; some being considered causal in instances of atherosclerosis and back pain. Inflammatory processes, long associated with AD, have recently been further elucidated, in particular revealing the role of the inflammasomes. We propose for a subset of AD patients, aging favors the overgrowth of oral anaerobes, established earlier in life, provoking a pro-inflammatory innate response that weakens the BBB allowing bacteria to spread and quietly influence the pathogenesis of AD. Finally, we suggest that human polymorphisms, considered alongside components of the microbiome, may provide new avenues of research for the prevention and treatment of disease.

Keywords: Alzheimer's, BBB, environmental, epidemiological, immune-tolerated, inflammasome, innate, microbiome, oral, polymorphism

ABBREVIATIONS

AD	Alzheimer's disease
Aβ	amyloid-beta
ApoE	apolipoprotein E
ASC	apoptosis-associated speck-like protein containing a CARD
BBB	blood-brain-barrier
CARD	Caspase activation and recruitment domain
Epha1	ephrin type-A receptor 1
F. nucleatum	*Fusobacterium nucleatum*
GWAS	genome wide association study
IL1β	interleukin 1-β
LPS	lipopolysaccharide
LOAD	late onset Alzheimer's disease
NGF	nerve growth factor
NLRC-4	NLR family CARD domain-containing protein 4
NLRP1-3	NLR family pyrin domain containing proteins 1-3
PCR	polymerase chain reaction
PKR	protein kinase RNA-activated
P. gingivalis	*Porphyromonas gingivalis*
TMAO	trimethylamine-N-oxide
TNFα	tumor necrosis factor α
TREM2	triggering receptor on myeloid cells 2

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INTRODUCTION TO THE MICROBIOME AND DISEASE

The human ecosystem

The human body plays host to a plethora of different microscopic organisms ranging in size and complexity from viruses and bacteria to multicellular, eukaryotic parasitic worms. However, this review will refer predominantly to the bacterial component of the microbiome. This accounts for roughly 1 – 3% of body mass with 10 bacteria for every human cell (NIH Human Microbiome Project) and bacterial load is likely to increase with age [1]. Bacteria are found in greatest numbers and variety in the mouth, the gut and on the skin.

Bacteria in the mouth, the gut and on the skin form biofilms. This is a complex ecosystem of different species of bacteria forming a symbiotic whole, enabling the attachment and proliferation of individuals [2]. Biofilm-forming bacteria release a highly hydrated matrix of extracellular polymeric substance, composed of proteins, polyuronic acids, nucleic acids and lipids. Together bacteria and this matrix form the bulk components of biofilm [3]. Of the estimated 700 oral bacteria identified by DNA, only around 50% have been cultured [4]. Many are "unculturable" probably because they cannot survive in isolation, but need other species for attachment and/or nutrients [2]. This is a common feature of biofilm "collectives".

The alimentary tract is a continuous tube running from the oronasal cavity to the anus. Commensal oral and gut bacteria metabolize components of the food we eat and release compounds which can be absorbed into the bloodstream. Hence, they contribute, with good and bad effect, to the chemicals circulating around our bodies. The bacterial composition of the entire tract is also likely to be influenced by diet as many foodstuffs e.g. garlic are antibacterial [5] or biofilm disrupters e.g. cranberries [6]. The old adage "you are what you eat," may have particular relevance in this context.

Another important feature of the alimentary tract is the immune tolerance afforded to bacteria residing in this location [7]. This is a necessary trade-off because immune surveillance must ignore foodstuffs to enable the continued survival of the host. However, this does present a potential hazard if any of our "commensals" migrate from their normal site of residence. Sensitive DNA analysis increasingly reveals that they do, and mounting evidence reveals that protective barriers such as the blood brain barrier (BBB) [8–10] and placenta [11] fail to provide comprehensive protection. It is also worth noting that when these barriers are breached by sub-acute levels of "immune-quiet" oronasal or gut bacteria [7, 12] they fail to elicit the discomfort associated with diseases such as meningitis or encephalitis [9]. However, outside their preferred environment there may be an accumulation due to immunosenescence [13]. The microbiome in the context of the aging immune system is discussed below.

The composition of the microbiome is influenced more by environmental and social factors than the host's genetic background as illustrated by a study of identical twins [14]. This study of the salivary microbiome showed that, having shared the same womb and home, monozygotic twins began life with very similar microbiomes which diverged as they led more independent lives. This is particularly relevant in the context of data for identical twin pairs discordant for disease as discussed in the epidemiological data below linking Alzheimer's disease (AD) to oral bacteria.

EXAMPLES OF THE MICROBIOME LINKED TO DISEASE

Atherosclerosis risk and the microbiome

Red meat consumption has long been implicated as a risk factor for atherosclerosis even when the meat consumed was lean and low in cholesterol. Trimethylamine-N-oxide (TMAO) forms part of the cascade to atherosclerotic plaque development and raised levels in blood act as a biomarker for atherosclerosis risk [15]. TMAO is produced as a metabolite of L-carnitine and other compounds such as phosphatidylcholine derived from lecithins abundant in red meat. Recently a link has been made between red meat/carnitine consumption and certain gut bacteria and demonstrates how diet influences the gut microbiome. Vegans and vegetarians produce less TMAO than omnivores fed L-carnitine [15]. This suggests that people with a low red meat diet have fewer of the specific bacteria required for TMAO production than those eating red meat more regularly. Studies in mice have shown that gut bacteria are required to metabolize carnitine and lecithin to produce TMAO for the progression of diet-induced atherosclerosis [16]. Mice raised in sterile conditions, or given antibiotics and then fed on a diet rich in carnitine and lecithin produced significantly less

TMAO [15–17]. This provides an example of how diet influences the composition of the microbiome and how components of the commensal microbiome contribute to developing a disease such as atherosclerosis.

Specifically, components of the oral microbiome have been implicated in atherosclerosis and stroke risk. The oral microbes *Aggregatibacter actinomycetemcomitans,* a facultative organism able to live aerobically or anaerobically and *Porphyromonas gingivalis (P. gingivalis)*, an obligate anaerobe, are implicated in atherosclerosis [18]. In the Zaremba *et al*. study the most frequently identified bacteria were *P. gingivalis* and *Treponema denticola* [19]. Other infectious agents such as *Cytomegalovirus* and *Chlamydophyla pneumoniae* are also associated with atherosclerosis [20]. Antibiotic treatment for cardiovascular disease has been largely unsuccessful, suggesting that a more long-term approach may be required to modify the background bacterial load. This is supported by another study showing that prolonged periodontal treatment that changed oral hygiene habits, successfully reduced oral anaerobes, reduced inflammatory biomarkers and even reversed thickening of the carotid artery intima-media, a known risk factor for stroke [21].

Cancer and the oral microbiome

Oral bacteria have long been associated with cancer. A recent prospective study found that the presence of serum antibodies to *P. gingivalis* increased mortality rates in orodigestive tract cancers, even in the absence of overt periodontitis [22]. Oral bacteria may be more than simply opportunistic organisms taking advantage of a compromised immune system to thrive, but may be actively and adversely affecting disease outcome, promoting tumor progression and metastasis. Potential mechanisms are now being explored. *P. gingivalis* has been found in oesophageal squamous cell carcinoma (OECC) tissues and identified by immunohistochemistry and quantitative RT-PCR to the 16S rRNA gene in 61% of OECC tumours (including 12% of adjacent tissue), but absent from all healthy tissue tested, whether from cancer patients ($n = 100$) or controls ($n = 30$) [23]. Another study shows how *P. gingivalis* may promote the invasion of healthy tissue by oral squamous cell carcinoma cells. *P. gingivalis* (but not *Fusobacterium nucleatum* (*F. nucleatum)*) induces the expression of proMMP9 by interacting with PAR-2 on tumor cells and then processes secreted proMMP9 to the active metalloprotease with its own gingipain proteases [24]. *F. nucleatum* has been detected in pancreatic tumors and this is also associated with poor prognosis [25]. One mechanism may be linked to immune subversion. *F. nucleatum* (from adenocarcinoma) interacts with Natural Killer (NK) immune cells to protect tumor cells from attack. The bacterial Fap2 binds to the human inhibitory receptor (TIGIT) on immune cells preventing the immune cells' killer response [26]. Perhaps in the future biopsy samples will be more routinely tested for bacteria and this may lead to new treatment regimens.

Low birth weight and preterm babies and the microbiome

Hormonal changes in pregnancy make women more prone to oral bacterial overgrowth leading to increased prevalence of gingivitis and periodontitis [27, 28]. During pregnancy, mothers with gum disease are more likely to give birth prematurely or to a low birth weight baby [29–31]. Not all studies have agreed on the degree of risk to pregnancy afforded by gum disease, suggesting that there may be variation between populations [31]. Animal studies have shown that oral bacteria cause low birth weight and prematurity [32, 33]. In most of the cases of human pre-term, low birth weight pregnancies examined, oral or gut bacteria were found by culture or DNA analysis to have crossed the placental barrier [34]. Growth retardation may result from primary effects on fetal development or as a secondary effect by impeding placental blood flow. Interestingly, among the bacteria identified that had crossed the placental barrier were the oral anaerobes *F. nucleatum* [33] and *P. gingivalis* [34].

Type II Diabetes and the microbiome

Clinical trials for Type II diabetes targeting oral bacteria show distinct differences between populations. In developing countries such as India and Brazil where obesity is less prevalent, treatment for periodontitis improved glycaemic control [35, 36]. Similar results were seen in Saudi Arabia where glycaemic control improved after periodontal treatment, but only significantly in combination with the tetracycline antibiotic doxycycline [37]. However in the United States (US) a large trial providing periodontal treatment to Type II diabetic patients showed no improvements in glycaemic control [38]. It is perhaps worth noting that the US trial did not use

systemic antibiotics and no information was available regarding the Body Mass Index of patients. It therefore remains possible that in the US population there may be a greater contribution to Type II diabetes by bacteria further down the gastrointestinal tract. Recent metagenomic approaches showed altered gut microbiota in Type II diabetics [39]. In addition to the serological prevalence of bacterial infections [40], evidence for local immune responses and occurrence of various bacteria in the affected pancreatic islets was also reported [41]. Antigens specific to *Helicobacter pylori* and *Chlamydophyla pneumoniae* as well as spirochetes were detected in lesion sites and it was suggested that oral and intestinal spirochetes may be candidate pathogens for Type II diabetes [41].

Obesity is an established risk factor for Type II diabetes [42] and has long been linked to the overgrowth of certain gut bacteria [43]. More recently a study published in Science explored the difference between the gut microbiomes of pairs of identical and fraternal human twins discordant for obesity. Astonishingly this work revealed that obesity was transmissible [44]. Gut bacteria cultured from each twin were fed to mice reared in a sterile environment, with no developed microbiome of their own. The mice receiving bacteria from the obese twins ("fat" bacteria), developed obesity and those receiving bacteria from the thin twins, ("thin" bacteria) remained normal weight, even though all mice were fed the same amount of food. The conclusion from these studies was that it is the combination of having a predominantly "thin" flora and being fed a healthy diet that is important for maintaining a normal weight. A poor diet, lacking fruit and vegetables will likely result in the "thin" bacteria being out-competed by any available "fat" bacteria [44].

EXPLORING THE LINKS BETWEEN ALZHEIMER'S DISEASE AND ORAL BACTERIA

Background for Alzheimer's disease

Dementia affects one in 14 people over 65 years of age and one in six people over 80 years of age in the UK. According to UK figures for 2012 published by the Alzheimer's Society, AD is the most common form of dementia accounting for 62% of cases (of the remainder, 17% have vascular dementia (VaD), 10% have mixed dementia (AD and VaD), 4% have dementia with Lewy bodies, 2% have frontotemporal dementia, 2% have Parkinson's dementia and 3% have other dementias). It is possible that the links between the oral microbiome described here apply to other forms of dementia but for the purposes of this review we focus on AD as it is the most common form.

AD symptoms frequently begin with loss of ability to form new memories, eventually leading to confusion. Ultimately, inability for self-care often results in institutionalization. There are now approximately 500,000 AD sufferers in the UK and 5.4 million in the USA, costing approximately £15bn and $183bn respectively per year [45]. Dementia is age-related and in Western Europe and the US prevalence (estimated by meta-analysis) in the 85–89 age range is 22%, rising to 40–50% for the over 90s. There is variation between populations, for example figures for Latin America show higher dementia rates of 28% for the 85–89 year olds and 64% for the over 90s [46]. This places a huge burden on society in terms of economics and human suffering especially in the context of a burgeoning, aging population. There is currently no cure, but it has been calculated that any intervention capable of delaying the symptoms of late onset Alzheimer's disease (LOAD) by even five years, would almost halve the projected number of new cases [47].

The neuropathological changes associated with AD are similar for familial and sporadic forms of the disease. However familial AD results from autosomal dominant mutations in the genes encoding either the amyloid-β protein precursor or presenilin proteins which accelerate the onset of cognitive decline, often affecting people in their forties or fifties. AD affects many areas in the brain including the hippocampus, frontal, temporal and parietal cortices and the cholinergic basal forebrain. The distinctive neuropathological features, amyloid plaques and neurofibrillary tangles are found in these areas at much higher density than observed in the brains of age-matched cognitively normal subjects. The hippocampus, entorhinal and transentorhinal cortices and basal forebrain are particularly vulnerable early in the disease trajectory. In these areas neurofibrillary tangles form and amyloid-β (Aβ) peptides are deposited as amyloid plaques [48, 49], neurites withdraw and synapses are lost, eventually resulting in cell death. The neurotrophins especially nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) are required here for maintaining cell viability and synapse connectivity and the availability of both diminishes as AD progresses [50].

Neurofibrillary tangles are intracellular fibrillar deposits of hyperphosphorylated tau proteins (Fig. 1A). Amyloid plaques are extracellular deposits of predominantly fibrillar Aβ peptides (Fig. 1B). Aβ peptides are derived from cleavage of the amyloid-β protein precursor by the enzymes β-secretase and γ-secretase. Cerebrovascular amyloid deposits are found in the small blood vessels of the leptomeninges and cortices of around 80% of AD brains.

Figure 1A shows neurofibrillary tangles comprised of hyperphosphorylated tau. Figure 1B shows an amyloid plaque comprised of mostly fibrillar Aβ.

The role of inflammation in Alzheimer's disease and cognitive decline

There is evidence of an inflammatory response within the AD brain. Glial cells such as astrocytes are recruited to sites of inflammation and once activated, become hypertrophic and contribute to the inflammatory processes by releasing pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1-β (IL1β). Structures known as inflammasomes form within innate cells e.g. microglia, in response to intracellular pathogens and trigger a process known as pyroptosis. This is an inflammatory form of programmed cell death. (It is worth noting here that at least one oral species of bacteria, *P.gingivalis,* is able to invade host cells [51].) Inflammasomes are comprised of cytoplasmic components including cryopyrins (NLR family pyrin domain containing 3 (NLRP1-3)), nod like receptors e.g. NLR family CARD domain-containing protein 4 (NLRC-4), the adaptor protein (apoptotic-associated speck-like protein containing a CARD (Caspase activation and recruitment domain) (ACS)), and caspase-1. Assembly is triggered by several different mechanisms including ligand-mediated toll-like receptor activation or double-stranded RNA dependent protein kinase (PKR) activation. The net result is the production of IL1-β release in response to e.g. bacterial lipopolysaccharide (LPS) [52]. *P. gingivalis* has been shown to activate the inflammasome pathway and triggers the release of IL1β via caspase-1 in immune cells [53]. The interplay between the inflammasome and autophagy within microglia potentially plays a role in the pathogenesis of neurodegenerative diseases. One study has shown that inhibiting autophagy with genipin (a small molecule found in gardenia fruit) has a knock-on effect of inhibiting downstream inflammasome activation [54]. Further investigations are warranted to determine how Aβ, normally cleared via the autophagy route, in its various oligomeric and fibrillar forms, affects this interplay.

Activated astrocytes also produce ApoE which may be involved in Aβ fibrillisation. Over a period of months or years the cycle of continued release of pro-inflammatory cytokines and amyloidosis exacerbates neuronal damage. It is noteworthy that systemic inflammation is also associated with confusion and raised serum levels of Il-6 have been implicated in post-operative delirium risk in the elderly [55].

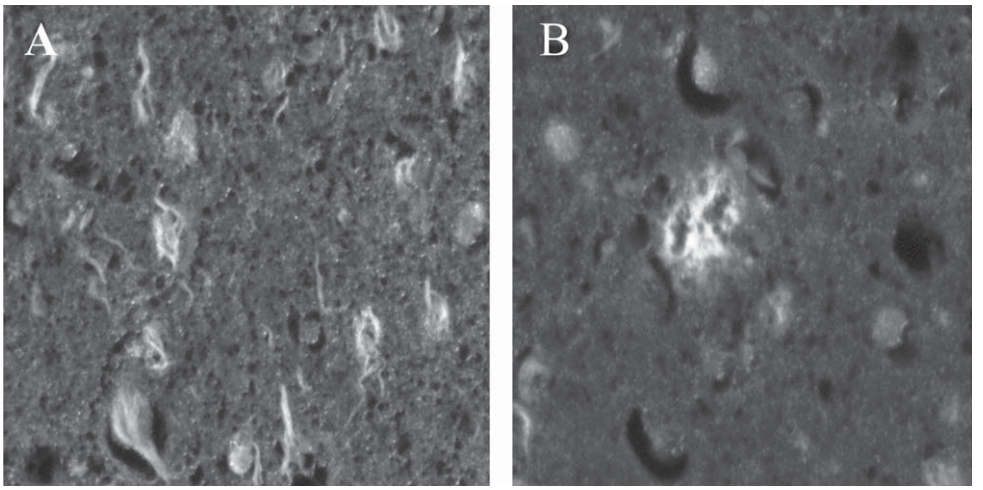

Fig. 1. Thioflavin S stained temporal slice from the brain of an AD patient.

Epidemiological links between oral bacteria and dementia

The Swedish Twin Registry [56] was set up in the 1950s and chronicled the life and medical histories of twins, including about 20,000 monozygotic pairs born between 1886 and 1967. One of the more surprising correlations to emerge from the data for these identical twins was that of dementia with tooth-loss in early to mid-life [57]. Of the three potentially modifiable risk factors, tooth-loss before age 35, poor education and short adult stature, only tooth loss was statistically significant in the identical twins discordant for dementia. Bearing in mind identical twins that live apart are unlikely to share the same oral microbiome [14], this emphasises a potential link between oral hygiene and dementia risk.

In accord with this, a twelve year study of North American Nuns reported a similar correlation between tooth loss and AD [58]. The cohort comprises of nuns who provide a vast amount of medical and personal history and donate their bodies to science for post-mortem study. This enables past medical and social history to be correlated with brain pathology. Notably, pre-existing tooth loss was shown to carry an odds ratio of 2.2 for developing LOAD [58].

Assuming that tooth loss provides a rough indicator for poor oral hygiene this link was further corroborated by an eighteen year longitudinal study from the US. Dentate individuals who did not brush their teeth daily were reported to have a 22 to 65% greater risk of developing dementia compared with those who brushed their teeth three times daily [59].

EXPERIMENTAL EVIDENCE LINKING ORAL BACTERIA TO ALZHEIMER'S DISEASE

Evidence of bacteria found in brains

Miklossy's work in the 1990s highlighted the involvement of several types of spirochetes in AD including oral, intestinal or as-yet uncharacterized species, as well the tick-borne Borrelia burgdorferi [60, 61]. In 2011 Miklossy published a review indicating that oral bacteria were present at ∼ 7-fold higher density and far greater variety in AD brains compared to cognitively normal controls. Among the AD patients examined, the most prevalent class of bacteria were oral spirochetes that are obligate anaerobes [9]. Previously Riviere and colleagues had used polymerase chain reaction (PCR) technology and species specific antibodies, to look for oral anaerobes (phyla Treponema) in brain samples [10]. PCR identified Treponema in 14 out of 16 AD brains compared with 4 of 18 controls with more species represented in AD. Treponema were also detected using antibodies in 15 out of 16 AD brains compared with 6 of 18 controls and there were significantly more AD subjects with cortical Treponemas compared with controls [10]. Riviere also examined trigeminal ganglia for bacterial infiltration by PCR. Treponema were detected in all subjects, although only samples from AD patients had *Treponema maltophilum* [10]. In order to establish the prevalence of bacteria in brains generally (non-AD), Branton and colleagues used deep sequencing with primers designed to amplify bacterial 16S ribosomal RNA genes [8]. They found evidence of bacteria across the samples tested, 70% of which were α-proteobacteria more normally found in soil and water, some of which have now been identified as part of the oral flora [62, 63].

Taken together, these findings suggest that certain bacterial phyla, in this case oral anaerobes, are more closely associated with AD, since they were not as heavily represented in the non-AD samples [8–10]. This is consistent with evidence of LPS from the oral anaerobe *P. gingivalis* in the brains of AD patients and not controls [64].

The link between bacteria and AD-like neurodegeneration has been further illustrated in a mouse model. The AD11 mouse produces antibodies which sequester NGF throughout life, steadily removing support for the cholinergic cells of the basal forebrain [65]. The adult AD11 mouse develops impaired memory function, Aβ and hyperphosphorylated tau lesions, loss of cholinergic basal forebrain neurons and hypertrophic ventricles in common with human AD [65]. Crucially, when these AD11 mice are raised in sterile conditions the onset of neuropathological changes and cognitive deficits is significantly delayed [66].

Evidence of oral bacteria and TNFα in blood in Alzheimer's disease

The association between raised TNFα and AD is well-established and in 2009 researchers in the US took blood samples from AD patients and cognitively normal control subjects. They used standard Enzymatic Linked ImmunoSorbent Assay (ELISA) techniques with antibodies to detect TNFα and looked for serum antibodies for the

periodontal bacteria *Actinobacillus actinomycetemcomitans, Tannerella forsythia* and *P. gingivalis*. Levels of TNFα and antibodies for oral bacteria were higher in AD patients compared to controls and the presence of serum antibodies for these bacteria carried an odds ratio of 6.1 (*p* value 0.04) for AD. The researchers suggested this could be used as a diagnostic tool [67]. Further to this, one longitudinal study has explored the potential for using oral bacteria in blood as a predictive tool. This study involved 158 people from the Biologically Resilient Adults in Neurological Studies research program at the University of Kentucky who were all cognitively normal at baseline. Raised baseline serum antibody levels, specific for the oral anaerobes *F. nucleatum* and *Prevotella intermedia*, correlated with cognitive deficits in subjects ten years later [68].

Important Implications for Alzheimer's disease progression

Whether oral bacteria themselves or endotoxins (e.g. LPS) released by them gain access to the brain, the net result is likely to be microglial activation. Microglial activation is a well-recognized feature of AD and results in the increased production of proinflammatory cytokines such as TNFα and IL1β. This could explain why levels of e.g. TNFα in the cerebrospinal fluid of AD patients reach such high levels, 25-fold that of controls [69]. As mentioned, prolonged exposure to high concentrations of TNFα weakens the protective BBB making it more permeable to ingress of e.g. bacteria or endotoxins [70].

It may be worth noting that cultured neuronal cells challenged with spirochetes produce Aβ [71] and that cultured neuronal (SH-SY5Y) cells exposed to LPS from bacteria, produce hyperphosphorylated tau [72]. We know that high concentrations of Aβ, oligomers, or fibrils, are neurotoxic. Research shows that Aβ is also toxic to bacteria and it has been suggested that this response may have evolved as part of the brain's defence against infection [73]. This would certainly help to explain the frequently observed amyloid deposition in cognitively normal brains, albeit at lower density. If the invading bacteria were susceptible to an Aβ response and the infection was successfully cleared, then one might expect some insoluble amyloid plaques to remain as testament to an infection successfully resolved. If however AD is caused or worsened by bacteria that provoke, but are not killed by, an Aβ response then we might expect both the focus of infection and the amyloid deposition to spread. It is also interesting to note that the same stain (congo red) which visualises bacterial beta pleated sheet formation (curli fibres on bacteria) is that which stains amyloid. It is suggested that the beta pleated sheet of amyloid triggers activation of the Toll-like receptors on microglia with subsequent inflammasome formation normally associated with bacterial invasion [74].

The genetics linking Alzheimer's disease to oral hygiene

There are several gene polymorphisms that have been associated with increased risk for sporadic AD. The ApoE4 polymorphism is most highly correlated with risk of LOAD. Again there is some population variation, but in a Norwegian study homozygosity for ApoE4 gave an odds ratio of 12.9, compared to 4.2 for ApoE3/E4 heterozygotes [75]. Among the many other detrimental effects with which it is associated, ApoE4 compromises the integrity of the BBB by activating the cyclophilin A matrix metalloproteinase MMP-9 pathway [76]. If bacterial or LPS entry into the brain plays a part in the initiation or progression of AD then maintaining an intact BBB is vital.

All other gene polymorphisms discovered so far, carry a lower individual risk for AD. The following section describes four genes associated with AD risk, immune function and bone homeostasis: the vitamin D receptor, TNFα, TREM2 and Epha1. The same polymorphism in the vitamin D receptor increases both risk of sporadic AD [77] and gum disease (periodontitis) [78]. TNFα is involved in immune function, BBB integrity and bone homeostasis. Polymorphisms that raise the expression of TNFα increase risk of AD [79] and periodontitis [80]. The triggering receptor on myeloid cells (TREM2) is expressed on microglia and is involved in immune function and bone homeostasis. A rare mis-sense mutation in the TREM2 gene (Rs75932628-T) confers risk for AD with an odds ratio of 2.9 in Iceland [81] and a similar risk has been reported in a Spanish population [82]. In bone homeostasis TREM2 acts as a co-stimulator, enhancing osteoclastogenesis which increases the rate of bone resorption [83]. A recent study has shown that TREM2 levels are higher in the peripheral blood of AD patients and correlate with AD severity [84]. The Ephrin Type-A Receptor 1 (Epha1) gene is one of the latest genes associated with AD risk to come out of the genome wide association study (GWAS) [85]. Epha1 is expressed on many cell

types including those in the immune system and is involved in diverse processes. It is expressed on the vascular endothelium and is downregulated shortly after an initial response to bacterial LPS, TNFα and IL1β. In blood vessels this is thought to promote immune cell extravasation [86], perhaps relevant to BBB integrity in AD. Epha1 has also been implicated in bone homeostasis in a GWAS looking at bone geometry and hip fracture risk [87].

Interestingly, all of these genes share roles in immune function and bone homeostasis, even ApoE is involved in bone metabolism although its precise role remains unclear. It is perhaps not so surprising that genes that participate in immune function are involved in contributing to AD risk. Inflammatory cytokines are associated with AD and these are produced principally by the innate immune system. If we speculate that infection may be directly involved in disease initiation or progression in a subset of AD cases, it seems reasonable to expect immune involvement in risk. That ApoE4, TNFα and perhaps Epha1 also influence BBB integrity is particularly important if the penetration of bacteria or LPS into the brain is involved in AD pathogenesis in these subjects. An interesting question is whether the shared role in bone homeostasis is relevant or merely coincidental. If oral anaerobes are involved in a proportion of AD cases, then bone homeostasis may play an active role in influencing the composition of the oral flora and hence AD pathogenesis. Figure 2 is a schematic suggesting how factors that increase bone resorption at the jaw, are likely to favor the proliferation of anaerobes in the oral microbiome and perhaps link tooth loss to AD risk.

Figure 2 Schematic to illustrate how bone homeostasis could influence the composition of the oral microbiome increasing the risk of initiation or progression to AD in a subset of patients. Factors promoting bone resorption increase periodontal pocket depth and this shifts the balance of the oral microbiome, favoring the proliferation of anaerobes.

Potential dietary influences over the oral/gut microbiome

Many of the dietary components that have been associated with reducing the risk of AD are antibacterial [5, 88–91]. Taken regularly in the diet they would spend time being deposited around the mouth throughout life, where they are likely to influence the composition of the oral microbiome. Further down the alimentary tract they are also likely to influence the gut flora. The Mediterranean diet has long been espoused as helping to prevent AD [92]. This diet is rich in foodstuffs with proven antibacterial activity such as garlic [5] and olive oil [88, 89]. Other foodstuffs with antibacterial activity such as curcumin [91], and honey [90] are also anecdotally purported to provide some protection against AD. Honey has peptides toxic to bacteria e.g. beedefensin1 [93]. Others like cinnamon contain potent antibacterial compounds [94] and also disrupt bacterial adhesion [95]. Resveratrol is a natural component of grapes (and found in red wine), blueberries, raspberries, and mulberries [96] that has been associated with longevity and is likely to be neuroprotective with regard to AD as a dietary component [97]. It is interesting to note that like many of the moderately protective gene polymorphisms resveratrol is also involved in bone homeostasis and immune modulation. One study showed that resveratrol effectively suppressed the loss of jaw bone and the release of proinflammatory cytokines in a rat model of periodontitis [98]. Another study showed resveratrol reduced *P. gingivalis* adhesion to endothelial cells of [99]. Many studies [100–103] have described the ability of essential oils, derived from culinary herbs and dietary components to disrupt biofilm and this

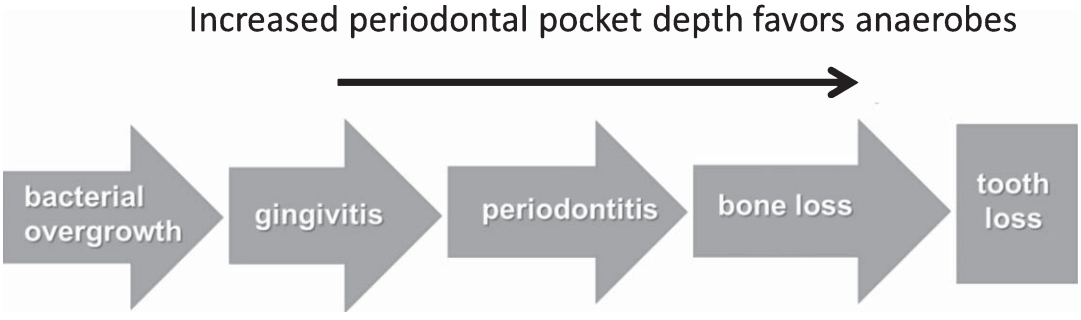

Fig. 2. Potential link between tooth loss and AD risk.

may be a key beneficial mechanism. Dietary beta glucans (β-glucans) such as provided by oats and mushrooms have generally been associated with providing health benefits e.g. in lowering cholesterol and immunomodulatory effects. Two studies in rats found that β-glucans successfully reduced loss of jaw bone in periodontitis [104]. The complex interplay between organisms within biofilm likely influences host cells. For example, *Candida albicans* increases host-cell invasion by *P.gingivalis* [105]. Dietary β-glucans may also modify oral biofilm composition which may reduce the influence of *P. gingivalis*. The use of β-glucans to treat severe periodontitis is currently in Phase 4 clinical trials.

A diet composed of many moderately beneficial foods may have subtle effects on the structure and composition of the oral and gut biofilms over a lifetime. Recent animal studies found that cinnamon extract added to the chow fed to a mouse model of AD, successfully reduced amyloid deposition and reversed cognitive decline [106]. Many of these "protective" foodstuffs also have anti-inflammatory properties previously thought to be beneficial in countering AD progression. As such, many have been tested for efficacy in clinical trials for AD with disappointing results. It is certainly worth noting that most, if not all, were administered in capsule form, by-passing the mouth entirely. They were therefore prevented from behaving as a foodstuff and rendered incapable of directly influencing the composition of the oral microbiome.

Why is the mouth a potential route to Alzheimer's disease?

The mouth connects a potentially hostile oral environment directly into bone via teeth, whilst at the same time affording immune protection for food and oral bacteria [7]. This must require carefully controlled immune surveillance. Immuno-tolerance may be particularly relevant if bacteria from the mouth (or gut) escape from their site of origin, allowing them to colonize new locations and quietly modulate host cell behavior. Oral epithelial cells produce TNFα in response to periodontal bacterial overgrowth and production is enhanced under anaerobic conditions [107], inferring that the anaerobes themselves stimulate an enhanced response. The capacity to produce TNFα is retained in old age [108].

Saliva is crucial in maintaining oral health. It performs many functions; lubricating contact between hard and soft surfaces, buffering plaque acids, re-mineralizing tooth surfaces as well as modulating the oral biofilm composition. The latter is achieved with the help of secreted antimicrobial agents such as immunoglobulins, histatins, peroxidases, lactoferrin and lysozyme [109]. Saliva is produced by the parotid, submandibular, sublingual, minor salivary glands and Von Ebner glands. The secretions in saliva are produced by mucus cells (sublingual and minor glands) or serous acinar cells (parotids) or both (submandibular). Each gland produces a secretion with a different composition. Saliva is produced constitutively as unstimulated flow or stimulated by the action of chewing [110]. The composition of stimulated and unstimulated saliva is not the same because each is composed of different proportions of secretions from the various glands and as such has different final constituents and properties. Adequate hydration is required for appropriate saliva production and many elderly people become poorly hydrated for many reasons. It is interesting to note that proton pump inhibitors, have been associated with increasing the risk for developing AD [111]. Omeprazole, among others in its class, also suppresses saliva flow [112].

Importantly as we age there is a general decrease in the production of unstimulated or maintenance saliva and this is further reduced by inactivity [113]. Saliva flow is influenced by posture and activity; greatest when standing, slower when sitting and further reduced when lying down [113]. Therefore the combined effects of aging, inactivity or infirmity, poor hydration and any medications that cause dry mouth, are likely to influence the oral flora and promote bacterial overgrowth in the mouth. It is also worth noting that saliva can only penetrate the oral biofilm to a certain depth, so its ability to influence biofilm composition is lost as oral hygiene deteriorates.

Potential route of entry for oral bacteria to the brain in Alzheimer's disease

Many nerves lead from the oronasal cavity directly to the brain, these include the trigeminal and olfactory nerves. The trigeminal nerve has been shown to harbor Treponema [10] and may act as a route of entry for oral bacteria into the brain in AD. Another potential route is the olfactory nerve, particularly in the context of hyposmia or anosmia as a heralding symptom for many neurodegenerative diseases, including AD [114]. The 'olfactory hypothesis', suggesting the olfactory tract as a potential route of entry for pathogens capable of triggering the production of amyloid plaques and neurofibrillary tangles, was first

introduced by Mann et al. in 1988 [115]. Olfactory ensheathing cells (OECs) provide bactericidal protection against invasion via the oronasal route. They share many of the capabilities of macrophages; they express inducible nitric oxide synthase when challenged, engulf bacteria and migrate [116]. However experiments have shown that bacteria such as *Staphylococcus aureus*, penetrate a compromised oronasal mucosa and arrive at the olfactory bulb within six hours, in spite of the release of proinflammatory cytokines [117]. OECs have been used successfully to deliver nanoparticles containing drugs to the brain, by-passing the BBB entirely [118]. OECs are able to engulf bacteria and migrate towards TNFα released by activated astrocytes [119]. Aged macrophages have impaired oxidative burst mechanism [120], if OECs senesce in a similar way, they could provide a vehicle for the transport of bacteria that are still alive.

Figure 3 highlights the similarities between AD disease progression and the route from the olfactory nerve to the hippocampus. One of the earliest symptoms in mild cognitive impairment that acts as predictor for the progression to AD is impaired olfaction [114, 121] and being unaware of this sensory deficit is considered more robustly predictive [122]. A pioneering study by Graves and colleagues explored hyposmia as a predictor of progression to AD in a normal elderly population of Japanese-Americans [123]. Assessments of 1,604 people were made at baseline and again, two years later. Anosmia at baseline carried a 1.92-fold increased risk for cognitive decline 2 years later and this risk increased to 4.9-fold if the anosmic person also carried at least one ApoE4 allele. This compares with a 1.23-fold risk for cognitive decline over the same time-period for normosmic people carrying one ApoE4 allele. The (sex-adjusted) risk for anosmic women with ApoE4 in this group carried a remarkable odds ratio of 9.7 compared to 1.9 with the ApoE4 alone [122, 123]. It may therefore be relevant that the olfactory bulb is the first site where neurofibrillary tangles and amyloid deposition is observed in the neuropathological trajectory of AD in humans [124] and mouse models of AD [125].

The next symptom that is often reported is when AD patients misrecognize faces that should be familiar [126]. The specific region responsible for facial recognition is the perirhinal cortex. A mouse model for AD has shown that Aβ deposits are observed in the perirhinal cortex early in the neuropathological trajectory [127]. The perirhinal cortex is connected to the olfactory bulb and also the hippocampus, the region responsible for forming new memories. Cell

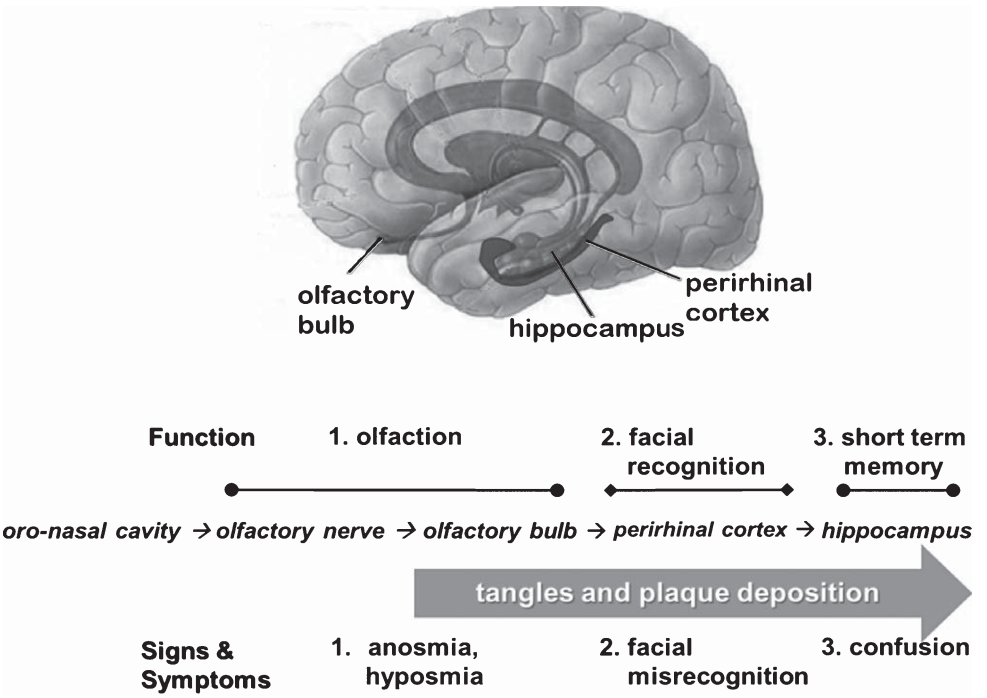

Fig. 3. Relationship between neuropathologically affected regions and signs and symptoms.

bodies in the hippocampus connect to cortical regions and maintenance of these connections is vital for the formation of new memories and cognition. Loss of these connections results in the confusion characteristic of AD.

Figure 3 illustrates the physical connection between the oronasal cavity to the hippocampus via the olfactory bulb, entorhinal and perirhinal cortices. The function pertaining to each brain region is noted above it and the signs and symptoms experienced by AD patients that also corresponds to the order of the appearance of neuropathological changes, are noted underneath.

CONCLUSIONS - FACTORS THAT LINK ORAL BACTERIA TO ALZHEIMER'S DISEASE IN THE CONTEXT OF AGING

The biggest risk factor for AD is old age, representing a one in four risk for the over eighties in Europe [46]. As we get older, bacterial load steadily increases as our humoral and cell-mediated immune responses wane in favor of the more primitive, but less efficient, innate immune system [13]. There is growing evidence that the microbiome composition, species identity and combinations, the density and distribution of these bacteria may influence how well we age. Gradually, as the innate immunity predominates over time, certain bacteria may proliferate and trigger more damaging responses. Against a background of rising bacterial load it becomes even more important to maintain the integrity of the BBB. Weakening of the BBB either, by any predisposing polymorphisms or as a result of conditions that elicit a sustained TNFα response, may serve to increase the propensity for bacteria or endotoxins to gain access to the brain, trigger neuropathology and alter brain function.

Many cell types provide innate immune support and are capable of releasing proinflammatory cytokines. These include cells in the oral epithelium which release more TNFα and IL1β in response to the bacteria that thrive as conditions become increasingly anaerobic [107]. Oral anaerobiosis is favored in gum disease due to increased periodontal pocket depth and for those wearing dental prostheses, such as dentures or bridges. We know the capacity to produce TNFα is retained throughout life [108], that levels are increased in inflammatory conditions including AD [67, 69] and that increased peripherally circulating TNFα weakens the integrity of the BBB [70]. Epha1 is one of the new genes from the GWAS associated with AD risk [85] and Epha1-mediated extravasation of immune cells from blood vessels is induced by TNFα [86]. OECs are immune cells which may be implicated in helping bacteria to track up nerves into the brain. It may therefore be informative to investigate a potential "Trojan horse" role for aging OECs under the influence of raised TNFα and determine whether Epha1 is involved.

The mouth is an excellent repository for low grade, chronic infection by bacteria that can flourish as the natural production of saliva diminishes with age, inactivity and as a possible drug side-effect. Immune tolerated oral bacteria [7] may escape into the circulation, lodge in other parts of the body and influence host cell behavior. Genetic polymorphisms that favor jaw bone resorption during inflammation will result in increased periodontal pocket depth. This provides the perfect habitat for the proliferation of the oral anaerobes, which are so far most closely associated with AD, to thrive. The oral microbiome may be particularly relevant to the high risk group identified by Graves and colleagues [123]. The odds ratio of 9.7 for LOAD specifically for women, with anosmia, who also carried one or more ApoE4 alleles was considered high. If the anosmia they experienced was due to oral anaerobes tracking up the olfactory nerve, perhaps the risk of progression to AD was increased if the BBB was also weakened by ApoE4. The association between tooth-loss in early to midlife in the twin and nun studies suggests oral bacterial overgrowth may be involved in a sub-group of AD patients. We know that hormonal fluctuations during the reproductive lifespan [128] and pregnancy [129] can exacerbate gum inflammation and induce changes to the oral flora. It is therefore possible that hormonal influences have provided conditions for the proliferation of pernicious oral bacteria that contributed to risk in the anosmic, ApoE4 positive women in the Graves study.

Clinical trials for intra-spinal injections of Etanercept (Enbrel), a TNFα sequestering antibody, have benefited some AD patients [130] and further clinical trials to assess the safety and efficacy of Etanercept as a potential therapy are ongoing. However, administration is invasive and if TNFα is produced in AD to combat infection, effects will likely be short-lived without addressing the underlying cause.

A clinical trial for the systemic antibiotics doxycyline and rifampin in AD patients has reported beneficial effects, slowing cognitive decline over 6 months [131]. Further evidence for bacterial involvement in AD is reported in a mouse model, as neurodegeneration is delayed when the AD11 mice

are raised in sterile conditions [66]. These results are encouraging and support the hypothesis that infection plays an active role in some cases of AD. The idea that infection causes the inflammatory responses in AD is not new, but maybe it is time to explore the possibility of a stealthy, low level infection by a "commensal on the loose". Furthermore this approach may be relevant to other neurodegenerative diseases. The task of modifying the oral or gut microbiome, once the reservoir for infection has been identified, will not be trivial. The bacterial species responsible are likely to differ between individuals, promoting the need for a tailored approach to both screening and treatment. Any intervention will need to provide a sustained, monitored reduction of the specific bacteria involved, having identified and encouraged the proliferation of beneficial bacteria within the microbiome. Among the (very) elderly, more people are likely to have an unhealthy diet and inadequate fluid intake. Further study into the effects of poor diet and hydration on the oral and gut flora may therefore be warranted in the context of cognitive decline. It may also be worth exploring whether influences on the oral microbiome help to explain the failure to translate the effects of some drug or dietary components from animal to human trials. If the mouth is a potential site of action, administering compounds in a form that allows prolonged oral retention may be beneficial (i.e. not swallowed in capsule form).

Modifying the oral microbiome will likely involve changing oral hygiene habits to disfavor harmful recolonization. If such interventions prove effective in slowing disease progression, they would provide an important route for a Public Health approach to reduce dementia risk in an aging population.

Looking forward, combining human genetic factors with microbiome composition could greatly improve our predictive capacity for assessing disease risk. Re-visiting the plethora of human genetic polymorphisms, which currently provide only weak indicators of risk, in the context of the microbiome may provide more accurate information. Furthermore combining these approaches could provide new opportunities for research into the prevention and treatment of disease.

This review was compiled using keyword searches in Google, Pubmed and Web of Science. Initial searches lead to subsequent keyword searches as the thread of the literature trail was followed down different pathways. Keywords included the following; Alzheimer's, aging, cognitive decline, microbiome, TNF-α, IL1- β, periodontitis, prevalence, gum disease, innate immunity, identical twins, discordant, tooth loss, inflammasome, resveratrol, terpenes, biofilm, autophagy, antibacterial properties, oral bacteria, blood brain barrier, olfaction, hyposmia, hormone, pregnancy, cycle, atherosclerosis, stroke, risk, gut microbiome, diabetes, obesity, low birth weight, pre-term, olfactory ensheathing cells, back pain, microglia, neuropathology, amyloid deposition, macrophage, hippocampus, perirhinal cortex, vitamin D, vitamin D receptor, TREM2, ApoE4, Epha1, saliva production, saliva components, diet, olive oil, curcumin, cinnamon, garlic, honey.

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Bacterial Infection Increases the Risk of Alzheimer's Disease: An Evidence-Based Assessment

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Abstract.
Background: The possibility of an infectious etiology for Alzheimer's disease (AD) has been repeatedly postulated over the past three decades, with the roles of both viruses and bacteria having been investigated. *Chlamydophila* (formerly *Chlamydia*) *pneumoniae* (Cpn) and spirochetal bacteria have been two of the most frequently implicated bacterial groups in AD pathogenesis.
Objective: A meta-analysis was performed where data were combined from 25 studies examining the association between AD and spirochetal bacteria or Cpn.
Methods: Comprehensive search of several electronic databases. Data were extracted from published studies and a random-effects model was used to analyze the data.
Results: A statistically significant association between AD and detectable evidence of infection of either bacterial group was demonstrated. Over a ten-fold increased occurrence of AD was noted when there is detectable evidence of spirochetal infection (OR: 10.61; 95% CI: 3.38–33.29), with a more conservative risk estimate demonstrating over a four-fold increased occurrence of AD (OR 4.45; 95% CI: 2.33–8.52). Over a five-fold increased occurrence of AD was noted with Cpn infection (OR 5.66; 95% CI: 1.83–17.51).
Discussion: There appears to be a strongly positive association between bacterial infection and AD.

Keywords: Alzheimer's disease, bacteria, *Borrelia*, *Chlamydophila*, dementia, etiology, infection, inflammation, spirochaetales, *Treponema*

INTRODUCTION

Alzheimer's disease (AD) was first described over a century ago and is the most common neurodegenerative disease, and yet an understanding of its etiology and pathogenesis remains elusive [1]. The worldwide prevalence of AD was estimated to be 26.6 million people in 2006 and it is predicted to quadruple by 2050, by which time 1 in 85 people worldwide will be living with this debilitating disease [2].

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AD is divided into two types, with an early-onset familial type associated with genetic mutations and a much more common late-onset form which is believed to be a multifactorial process that may involve infectious co-factors [3]. The possibility of an infectious etiology for AD has been repeatedly postulated over the past three decades, with the roles of both viruses and bacteria investigated. Evidence for a viral contribution is strongest for herpes simplex virus type 1 (HSV1), with the combination of HSV1 infection and carriage of the type 4 allele of the apolipoprotein E gene (APOE $\varepsilon 4$) found to be a strong risk factor for AD [4, 5]. In terms of bacteria, *Chlamydophila* (formerly *Chlamydia*) *pneumoniae* (Cpn) and spirochetal bacteria have been two of the

most frequently implicated bacterial groups in AD pathogenesis.

Cpn is a primary human pathogen which causes respiratory tract infections including bronchitis, pharyngitis and pneumonia and was officially identified as a separate species within the *Chlamydia* genus only relatively recently in 1989 [6]. The pathogen is transmitted via the respiratory route which is a key reason why its seroprevalence is relatively high at over 50% among adults in the U.S. and various other countries [7].

Spirochetes are helical Gram-negative bacteria that belong to the order Spirochaetales [8]. Syphilis caused by *Treponema pallidum* is one spirochetal disease that can involve cortical atrophy and dementia as late manifestations [8]. This has prompted researchers to investigate whether spirochetal infection could contribute to the development of AD in an analogous manner [9].

A number of case-control studies had examined whether there is an association between bacterial infection and AD, however conflicting results had not enabled a consensus to be reached. A study was therefore done to quantitatively assess all of the published data on the effect of bacterial infection upon the development of AD. The literature search yielded studies examining the relationship between bacterial infection and AD for various different bacteria. Quantitative data sufficient for meta-analysis, however, were found only for spirochetes and Cpn.

MATERIALS AND METHODS

Study protocol

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [10]. A systematic search of the databases MEDLINE (from 1950), PubMed (from 1946), EMBASE (from 1949) and Google Scholar (from 1993) was conducted through to January 2016, to identify relevant articles, using the two search terms 'Alzheimer's disease' and 'infection'.

Study selection

We included studies that met the following inclusion criteria: 1) there were data specific to AD as opposed to other or unspecified dementias; 2) AD was diagnosed by the appropriate clinical or neuropathological protocols; 3) appropriate laboratory methods were used to diagnose infection; 4) the risk point estimate was reported as an odds ratio (OR), or the data was presented such that an OR could be calculated; 5) the 95% confidence interval (CI) was reported, or the data was presented such that the CI could be calculated; and 6) an internal comparison was used when calculating the risk estimate. We excluded studies that did not meet these inclusion criteria. With regards to our second criterion, the majority of included studies involving living patients used the National Institute of Neurological and Communicative Disorder and Stroke-Alzheimer's Disease and Related Disorder Association (NINCDS-ADRDA) criteria for the clinical diagnosis of probable AD, which achieves the maximal certainty obtainable without an autopsy or biopsy [11]. Neuropathological examinations had been conducted to diagnose AD in studies of post-mortem brains, typically sourced from brain resource centers, with adherence to the neuropathological criteria developed by the Consortium to Establish a Registry for Alzheimer's disease (CERAD) specifically stated in many studies [12].

Statistical analysis

Pooled odds ratios and 95% confidence intervals were calculated for the effect of bacterial infection on the risk of AD using a random effects model [13]. We tested heterogeneity with Cochran's Q statistic, with $p < 0.10$ indicating heterogeneity, and quantified the degree of heterogeneity using the I^2 statistic, which represents the percentage of the total variability across studies which is due to heterogeneity. I^2 values of 25, 50, and 75% corresponded to low, moderate and high degrees of heterogeneity respectively [14]. Studies with extreme ORs were excluded in sensitivity analyses where appropriate in order to determine conservative risk estimates with lowered heterogeneity of results.

All analyses were performed with Comprehensive Meta-analysis (version 2.0, 2005; Biostat, Englewood, New Jersey).

RESULTS

Literature search

Of the 4039 references screened, we found 23 case-control studies, 3 case series and 1 randomized controlled trial eligible for inclusion in this meta-analysis, of which 13 studies concerned spirochetes and 14 concerned Cpn.

Study characteristics

The total numbers of AD and control cases were 723 and 481 cases respectively. Table 1 provides details of individual studies.

We found a significantly increased occurrence of AD when infection with either spirochetes or Cpn was detected.

AD and spirochetes or Cpn

Our analysis demonstrated over a ten-fold increased occurrence of AD when there is detectable evidence of spirochetal infection (see Fig. 1). The pooled odds ratio was 10.61 (95% CI: 3.38–33.29) although a moderate degree of heterogeneity was detected ($I^2 = 51.77$, $p = 0.02$). Four studies found to contribute to this heterogeneity were excluded in a sensitivity analysis to produce a conservative risk estimate (see Fig. 2) with an OR of 4.45 (95% CI: 2.33–8.52) [17–20]. No heterogeneity was detected in this conservative result ($I^2 = 0.00\%$, $p = 0.63$) and Egger's regression once again showed no publication bias ($p = 0.23$).

We found over a five-fold increased occurrence of AD when there is detectable evidence of Cpn infection (see Fig. 3). The pooled odds ratio was 5.66 (95% CI: 1.83–17.51) although notably a high degree of heterogeneity was detected ($I^2 = 73.42\%$, $p < 0.001$). Egger's regression showed no publication bias ($p = 0.28$).

Table 2 summarizes the key results of subgroup and other analyses performed, including assessment of the impact on the risk estimate of region, bacterial detection method and the material type tested.

DISCUSSION

What do the data imply?

The association between infection and AD was stronger in studies based on testing of brain samples compared to studies analyzing serum samples. Our findings suggested that infection with these bacteria increases the risk of developing AD. Although it remains unclear whether there is a cause and effect relationship or whether infection is a risk factor for AD, given the strength of associations found in our study, it is unlikely that infections with Cpn and spirochetes in the context of AD are coincidental findings.

One possible contribution to the development of the heterogeneity present in some of the results is the methodological differences between the studies meta-analyzed. For example, the material examined ranged from samples of brains, to sera and CSF. Further, there were differing detection methods utilized to diagnose infection, including PCR, IHC and ELISA. For both spirochetes and Cpn, studies assessing infection status based on examination of brain samples such as by PCR yielded considerably stronger associations with AD than serology-based studies. This is particularly significant because PCR analyses for bacterial DNA definitively establish the bacteria's presence in the brain, whereas serology-based findings of the presence or otherwise of antibodies cannot confirm or exclude bacterial presence in the brain. Further, serological testing is not performed for all types of spirochetes, and in fact such tests are lacking for the majority of oral spirochetes. This suggests that standardized detection methods would assist in developing more precise and accurate risk estimates.

Caution has to be used to avoid hastily equating correlation with causation of AD without clear evidence. The positive associations found in the study must be considered in the context of a number of studies having failed to find significant differences in Cpn and spirochete infection rates between AD and control cases [21–25]. The cause for the conflicting conclusions between these studies and others that have found very strong associations between infection and AD is likely to be multifactorial. Methodological differences between studies and the lack of standardized techniques are likely key factors. It has been postulated that one of the reasons why some groups have not had success in finding evidence of infection in AD brains is a low sensitivity of PCR analyses when sufficient replicate testing is not performed [26, 27]. Further, obtaining DNA of a sufficiently high quality for PCR from paraffin-embedded or other fixed tissue is notoriously more difficult than from frozen brain samples and this may also help to account for the diverse results given that some studies involved fixed tissue samples [26, 28].

Additionally, spirochetal and Cpn bacteria may be present only in small, focal regions of brains such that testing may yield negative results despite repeated and methodical testing of the same specimens [28]. Differences in DNA preparation such as whether proteases were used and differing cut-off values of immunoglobulin titers could also help explain the contradictory results [29]. In both the early and late phases of infection with the Lyme

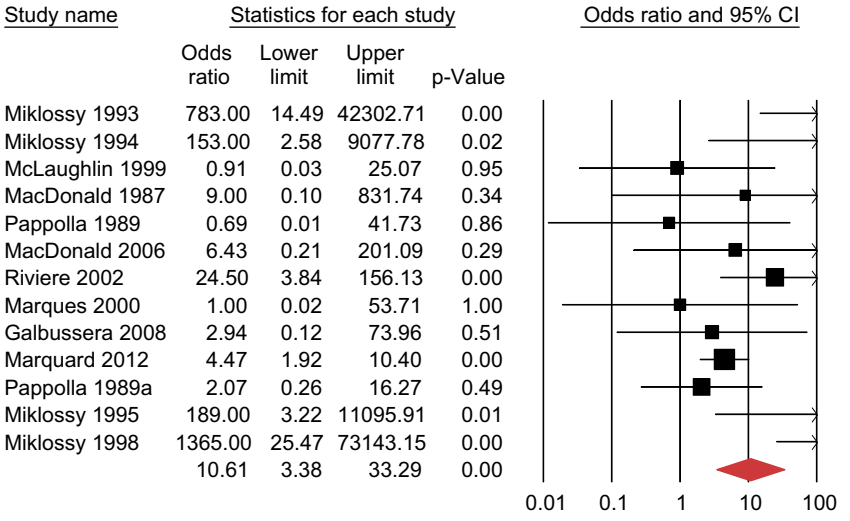

Fig. 1. Spirochetes and AD, a risk estimate with inclusion of all studies. The pooled odds ratio of 10.61 demonstrates a statistically significant association of spirochetes with AD ($p < 0.05$, $I^2 = 51.77$).

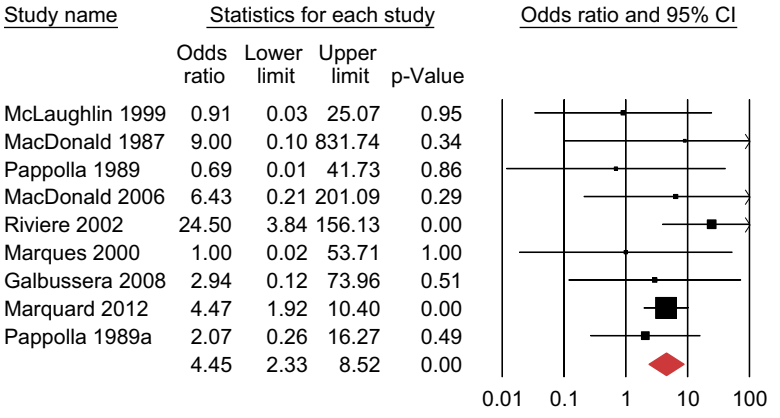

Fig. 2. Spirochetes and AD, a conservative risk estimate: the pooled odds ratio of 4.45 still demonstrates a statistically significant association of spirochetes with AD ($p < 0.05$, $I^2 = 0.00$).

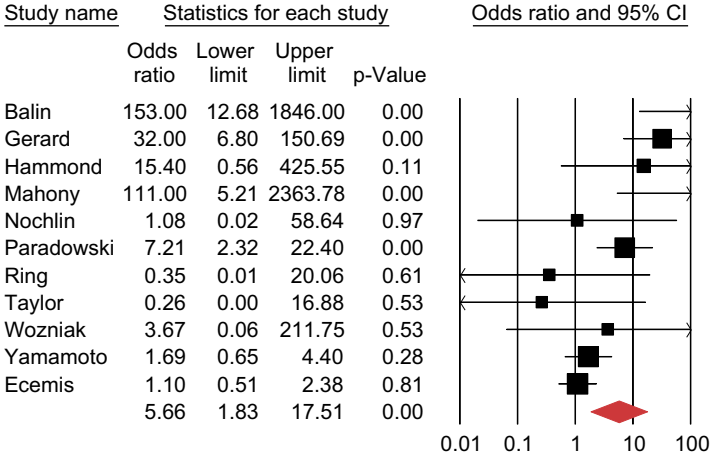

Fig. 3. Cpn and AD: the pooled odds ratio of 5.66 demonstrates a statistically significant association of Cpn with AD ($p < 0.05$).

Table 1
Study characteristics

First author, year [ref]	Total sample size	AD cases	Control cases	Detection method for bacteria	Material tested	Country
Spirochetes						
Case-control studies						
Miklossy 1993 [18]	27	14	13	DF, culture, EM	Cortex, serum	Switzerland
Miklossy 1994 [17]	12	8	4	DF	Cortex	Switzerland
McLaughlin 1999 [21]	28	22	6	DF, EM	Serum	Canada
MacDonald 1987 [9]	2	1	1	IHC	Cortex	U.S.A.
Pappolla 1989 [30]	10	6	4	EM, IHC	Cortex	U.S.A.
MacDonald 2006 [57] [58]	11	10	1	PCR, IHC	Cortex	U.S.A.
Riviere 2002 [59]	34	16	18	PCR	Cortex	U.S.A.
Marques 2000 [31]	30	15	15	PCR	Cortex	U.S.A.
Galbussera 2008 [22]	98	50	48	IFA	Serum	Italy
Marquard 2012 [60]	200	100	100	ELISA, Wbl	Serum	Germany
Pappolla 1989a [30]	47	16	31	ELISA, IFA	CSF	U.S.A.
Miklossy 1995 [19]	14	10	4	IHC	Cortex	Switzerland
Miklossy 1998 [20]	42	32	10	IHC	Cortex	Switzerland
Case series						
Gutacker 1998 [61]	27	27	0	ELISA, Wbl	Serum	Switzerland
Gutacker 1998a [61]	10	10	0	PCR, DF	Cortex	Switzerland
Chlamydophila pneumoniae						
Case-control studies						
Balin 1998 [36]	38	19	19	PCR	Cortex	U.S.A.
Gérard 2006 [26]	52	25	27	PCR	Cortex	U.S.A.
Hammond 2010 [62]	10	5	5	IHC	Cortex	U.S.A.
Mahony 2000 [27]	31	21	10	PCR	Cortex	Canada
Nochlin 1999 [23]	25	12	13	ICC, PCR	Cortex	U.S.A.
Paradowski 2007 [51]	104	57	47	PCR	CSF	Poland
Ring 2000 [24]	20	15	5	PCR	Cortex	U.S.A.
Taylor 2002 [25]	11	9	2	PCR, IHC	Cortex	U.K.
Wozniak 2003 [28]	20	4	16	PCR	Cortex	U.K.
Yamamoto 2005 [63]	93	61	32	ELISA	Serum	Japan
Ecemis 2010 [64]	104	54	50	ELISA	Serum	Turkey
Randomized controlled trial						
Loeb 2004 [50]	82	82	0	IFA	Serum	Canada
Case series						
Gieffers 2000 [65]	20	20	0	PCR, ICC	Cortex	Germany
Dreses-Werringloer 2009 [66]	2	2	0	PCR, culture	Cortex	U.S.A.

CSF, cerebrospinal fluid; DF, dark field microscopy; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; ICC, immunocytochemistry; IFA, immunofluorescence assay; IHC, immunohistochemistry; PCR, polymerase chain reaction; Wbl, western blot.

Table 2
Results of sub-group analyses

OR (& 95% CI) unless otherwise stated	Spirochetes (all studies, primary analysis)	Spirochetes (conservative, secondary analysis)	Cpn
Region: Europe	58.55 (5.63–609.12)	4.35 (1.92–9.85)	2.19 (0.52–9.19)
Region: North America	4.55 (1.53–13.53)	4.55 (1.53–13.53)	19.52 (3.82–99.75)
Detection method: dark field microscopy	41.86 (0.62–2843.94)	0.91 (0.03–25.07)	N/A
Detection method: ELISA	3.94 (1.84–8.41)	3.94 (1.84–8.41)	1.30 (0.71–2.38)
Detection method: IHC	37.40 (1.32–1063.28)	2.20 (0.11–45.89)	15.40 (0.56–425.55)
Detection method: PCR	11.02 (2.13–56.94)	11.02 (2.13–56.94)	9.95 (2.45–40.32)
Percentage of AD cases with combined infection detected (&95% CI): brain, CSF, sera examinations	38% (17–65%)	15% (5–33%)	50% (32–69%)
Percentage of AD brains with infection detected (&95% CI)	55% (18–87%)	23% (4–68%)	41% (14–74%)

disease-causing spirochete *Borrelia burgdorferi*, the antibody levels may be within normal limits thus suggesting that direct measurement of antigens within the brain may be needed to confirm serology results [22]. Thus, a standardized set of protocols and procedures for assessing infection status seems key to the

development of more definitive conclusions regarding the contributions of bacterial infections to AD pathogenesis. Another possible contributor to the diverse results in existing spirochetal studies is that a number of studies found AD and control cases to be negative specifically for the spirochete *Borrelia burgdorferi* whereas other spirochetes were not tested for [22, 30, 31]. Therefore, spirochetes other than *Borrelia burgdorferi* may have been present in a greater number of AD cases than in control cases. Their methodologies contrast to the methodologies used in two studies, which tested for all types of spirochetes [17, 18].

The amyloid cascade hypothesis of AD pathogenesis has been the most dominant hypothesis for AD and it describes the accumulation of the amyloid-beta peptide (Aβ) leading to neuronal death and dysfunction and consequently dementia [32]. While familial AD is known to be caused by genetic mutations resulting in increased amyloid accumulation, the late-onset form of AD (LOAD) has been shown not to directly arise from an identical or other genetic defect, thus making it likely that the pathogenesis of LOAD is a multifactorial process [32]. The type 4 allele of the apolipoprotein E gene (APOE ε4) is a strongly confirmed genetic risk factor for LOAD [33]. This genetic predisposition represents one factor, which may determine the outcome of infection with Cpn and spirochetes. Thus a synergistic action of bacterial infection with factors such as the carriage of APOE ε4 may cause the development of AD. Parallels may then be drawn between AD and other disease entities such as tuberculosis where microbes infect some people only asymptomatically and cause disease in other individuals due to other factors causing increased susceptibility to disease.

Apart from respiratory infections, Cpn has also been associated with chronic inflammatory diseases and atherosclerosis, although its exact role has been difficult to establish in most chronic disease contexts and thus its role has not drawn widespread support within the clinical and research communities [34]. A key basis for the chlamydial infection hypothesis for AD is that the organism can switch from an acute replicative phase to a state of chronic, latent infection, provoking neuroinflammation that precedes or coincides with the deposition of Aβ [3]. While Cpn infection can cause cell death by necrosis, it can also inhibit apoptosis and thereby sustain a prolonged neuronal infection and contribute to chronic inflammation in the brain [35]. Chronic infection in the AD brain may promote amyloidogenesis.

Many cell types in the AD brain have been found to be infected with Cpn of confirmed viability and metabolic activity, including monocytes, neurons, and glial cells [26, 36]. The latter may be evidence of infection-initiated inflammation contributing to AD pathology given that the stress response of glial cells involves the production of reactive oxygen species and pro-inflammatory cytokines [32]. Cpn infection of monocytes produces changes in monocyte gene transcription and sustained secretion of pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8 [37]. This aligns well with other epidemiological, clinical, and basic science studies which have implicated immunological responses more broadly in the pathogenesis of AD [38].

Cpn-infected cells have been found to co-localize closely with both neuritic senile plaques (NSPs) and neurofibrillary tangles (NFTs) [26]. Cpn has also been identified by immunohistochemistry in the olfactory neuroepithelia, bulbs and endothelia of mice and the brains of the mice inoculated with Cpn were shown to undergo Aβ deposition [39]. This suggests that the olfactory pathway may be a mode of Cpn entry into the central nervous system and that Cpn may be capable of accelerating or inducing AD-like pathology [39]. A higher Cpn load being found in the brains of ε4-carrying AD patients compared to non-ε4 carrying patients is significant given that carriage of APOE ε4 is a well-established risk factor for AD [40]. This suggests there is a link between Cpn infection, the product of the APOE ε4 allele, and AD.

Analogously to Cpn, spirochetes have been implicated in a number of chronic inflammatory conditions in body tissues other than in the brain, including periodontitis and ulcerative gingivitis [41]. It is widely accepted that chronic infections caused by spirochetes such as *Treponema pallidum* can cause chronic neuropsychiatric disorders including dementia. First investigated in 1913, it is now well-established that in a late-stage form of syphilis known as general paresis, *Treponema pallidum* causes dementia by inducing cortical atrophy, microgliosis, and amyloid deposition [42]. Dementia has also been reported to occur in Lyme disease, caused by the spirochete *Borrelia burgdorferi* [43]. Spirochetes have been found intracellularly within neurons and glial cells and capable of establishing chronic infection and causing cellular dysfunction and apoptosis [44]. Exposure of primary mammalian neuronal and glial organotypic cell cultures to *Borrelia burgdorferi* spirochetes was found to induce the pathological hallmarks of AD including

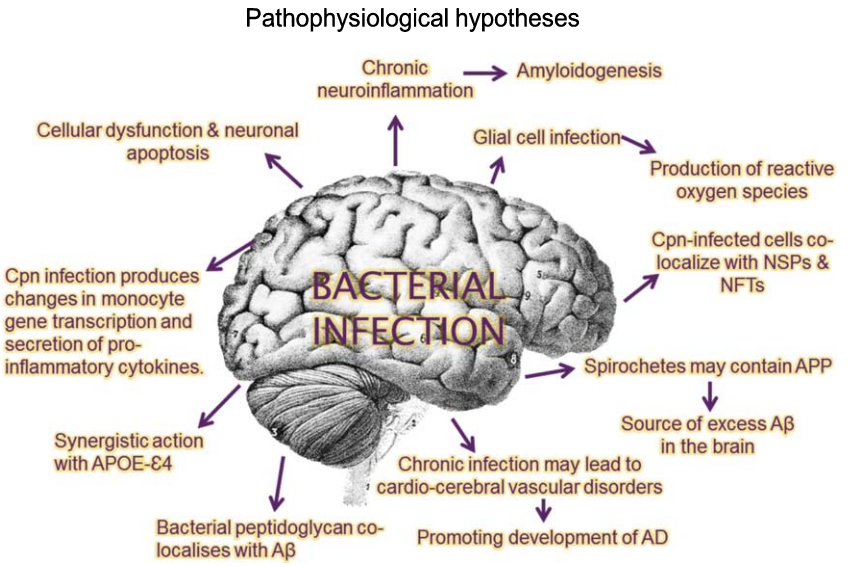

Fig. 4. Key evidence supporting the role of bacterial infection in the development of Alzheimer's disease (Source of image: Wikimedia Commons).

Aβ deposition, increased levels of amyloid-β protein precursor (AβPP) and hyperphosphorylated tau in the form of NSP- and NFT-like structures [45].

Miklossy found evidence of spirochetal infection in all 14 AD brains studied and in none of the 13 control brain tissue samples and also found that the spirochetes in the AD cases demonstrated positive immunoreaction with a monoclonal antibody targeted against AβPP [18]. This indicates that spirochetes may contain AβPP and thus the pathogens may be the source of excess Aβ in the AD brain [18]. A parallel can be drawn with HSV1, as AβPP has been an identifiable component of HSV1 intracellular viral particles, although it is unclear whether AβPP joins HSV1 particles *in vivo* or during procedures to isolate the virus [46]. Miklossy also described finding that bacterial peptidoglycan (an inflammatory and amyloidogenic cell wall component of bacteria including spirochetes) co-localizes with the Aβ in NSPs and NFTs [20]. Morphologically, the senile plaques were observed to be similar to spirochetal colonies in the cortex in established spirochetal disease [20]. Such observations collectively implicate spirochetal and Cpn infection in the development of the hallmark neuropathology of AD, although the exact mechanisms by which the bacteria may contribute to neuronal cell injury and death and Aβ accumulation continue to be investigated. Some of the key aspects put forward regarding the link between infection and AD are summarized in Fig. 4.

More recently, Bu et al. investigated the percentage distribution of infectious burden (as evaluated by measuring serum antibody levels) in healthy controls compared to AD patients. They found that 41% of AD patients versus 24% of healthy controls demonstrated seropositivity towards two or more of *Borrelia burgdorferi*, Cpn and *H. pylori* [47]. Further, AD patients and healthy controls with a higher infectious burden (comprising evidence of both viral and bacterial infections) were found to have higher serum levels of inflammatory markers such as IFN-γ, TNF-α, and IL-6, higher serum Aβ levels, and worse cognition. This study is notable in its design for assessing the infectious burden consisting of bacteria and viruses previously implicated in the development of AD as a whole group rather than focusing on one pathogen alone. Thus, not only has an increasing infectious burden been confirmed to be associated with AD, but through this study there is evidence that accumulative infections are associated with AD [47]. This synergistic effect of multiple infections was also noted in a recent study showing that seropositivity to both *H. pylori* and latent toxoplasmosis produced increased susceptibility to cognitive deficits than seropositivity to either infection alone [48].

While the number of studies directly assessing infections as risk factors for AD are comparatively few, it is well-established that AD patients experience accelerated cognitive decline in the setting of acute infection [49]. The results of further studies

assessing the role of infection in the development of AD could impact on the direction of future therapeutic strategies for management of AD. A randomized controlled trial showed that combination treatment for 3 months with antibiotics active against Cpn was found to reduce cognitive deterioration at 6 months of follow-up in patients with mild to moderate AD [50]. Further confirmation of the association between bacterial infection and AD is required before treatment with such antibiotics and/or anti-inflammatories of at-risk populations or following early diagnosis can be fully justified.

It has been suggested that an impaired blood-brain barrier in the AD brain may facilitate entry of bacteria thereby causing the differences in the positive results of AD patients and control cases rather than the bacteria contributing to the pathogenesis of AD [51]. However, Cpn for example was present in the post-mortem brain tissue of patients with prior chlamydial vascular infection and without noted AD, suggesting that infection may predate the development of AD pathology [52]. An important direction of future research would be to conduct prospective, longitudinal studies which would enable an observation of the temporal order of infection and AD development and to enable more definitive conclusions to be drawn on whether a causal relationship exists between AD and spirochetal or Cpn infection.

The importance of a standardization of the techniques and protocols used to assess infection with Cpn, spirochetes, and other bacteria in future studies is further highlighted given the inconsistencies within the existing literature. It is recommended that future studies use as controls only those cases without any identifiable AD-related pathological changes, as the use of controls with any degree of AD pathology may produce difficulties in interpreting results. The seropositivity for *Borrelia burgdorferi* is plausibly very low in the general population meaning that a very large sample size ideally needs to be recruited in order to develop sufficient statistical power to confirm the results of the present meta-analysis for that spirochete [22]. Strong positive associations have been reported between AD and infections with *Helicobacter pylori*, periodontal pathogens, and *Toxoplasma gondii* [53–56]. We advocate for further studies to be done to confirm these associations given the paucity of existing data for these bacteria. Demographic differences between patients groups including geographic location may be a factor in the inconsistent data on the association of bacterial infection with AD. The majority of existing studies originate from North America or Europe, so future studies conducted in other regions may provide further insight on the association between bacterial infection and AD.

CONCLUSIONS

The ageing of the global population means that the social and economic burdens associated with AD will grow alongside the dramatic increase in the number of people with AD. Over four to five-fold increased occurrences of AD were found with spirochetal and Cpn infection respectively, so we conclude that there is a strongly positive association between bacterial infection and AD. Though pathophysiological mechanisms whereby infection may contribute to the development of AD are yet to be definitively established and methodological differences have likely contributed to conflicting results between studies in the past, the overall analysis is suggestive of infection as a risk factor for the development of AD at the very least. The efforts to develop disease-modifying treatments will be served well by further testing of the bacterial hypothesis for AD to generate a better understanding of the pathophysiology of this disease.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/16-0362r1).

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Section 4
Periodontal disorders and Alzheimer's disease

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Alzheimer's Disease and Peripheral Infections: The Possible Contribution from Periodontal Infections, Model and Hypothesis

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Abstract. Alzheimer's disease (AD) affects approximately 5.3 million people in the U.S. and this number will increase as the population ages and the life-span increases. Therefore, of paramount importance is identifying mechanisms and factors that affect the risk of developing AD. The etiology and pathogenic mechanisms for AD have not been defined, although inflammation within the brain is thought to play a significant role. Consistent with this hypothesis, studies suggest that peripheral inflammations, dysbiotic conditions, and infections contribute to the inflammatory state of the brain and may constitute risks for AD. Recently, several peripheral conditions with an inflammatory basis such as diabetes and obesity have been recognized as risks for AD. Periodontitis is a prevalent, chronic peripheral polymicrobial disease associated with gram negative, anaerobic bacteria, which exhibits significant localized and systemic inflammatory effects. This review will present evidence suggesting that periodontal disease may also be a risk factor for AD and possible mechanistic links between periodontitis related inflammation and AD. It will review the pathogenesis of periodontitis and the mechanisms by which periodontal infections may affect the onset and progression of AD. Periodontitis is a treatable condition and may be a readily modifiable risk factor for AD. Therefore, further studies including intervention trials are warranted.

Keywords: Alzheimer's disease, peripheral infection, inflammation, dysbiosis, periodontitis, periodontal bacteria, cytokines

INTRODUCTION

Alzheimer's disease (AD) is one of the most common causes of dementia in elderly populations [1], afflicting approximately 5.3 million people in the United States. Although the rates of prevalence and incidence vary among study populations, these rates increase significantly with age [2]. It is projected that by 2050, as the population ages and the life-span increases, AD will afflict approximately 14 million people in USA and 115 million world wide [3]. It is therefore clear that AD constitutes an increasing public health concern. However, the prevalence of AD

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will not change significantly unless new treatments emerge that can prevent, reverse, delay the onset or slow the progression of the disease. It is estimated that delaying the onset of AD by 5 years could result in 50% decrease in its prevalence in 50 years. Susceptibility to develop AD is dependent upon genetic and environmental factors [4]. While some AD risk factors are immutable others may be modifiable and therefore may constitute a means to significantly limit the prevalence of this disease in the future.

While the specific factors involved in the etiology and pathogenesis of AD are not well characterized, it is accepted that inflammation plays a significant role. Its role can be primary [5, 6], secondary or a combination of both. A central tenet of the inflammatory hypothesis is that peripheral processes alter brain inflammation. Studies have shown that peripheral infections can hasten the onset and progression of AD through an inflammatory mechanism [7–11]. Periodontal disease (PerioD) is a common, chronic, peripheral, inflammatory, polymicrobial disease that has been linked to other systemic inflammatory conditions [12, 13]. The objective of this review is to present possible mechanistic links between PerioD and AD. It will offer evidence in support of a model explaining the initiation and maintenance of inflammation in AD and associated progressive AD related pathology. According to our model, periodontal bacteria induce pro-inflammatory cytokines and C-reactive protein (CRP) which stimulate glial cells to produce Aß amyloid 1–42 peptide (Aß42) and hyperphosphorylated tau protein (P-Tau), which consequently induces production of more inflammatory molecules. Thus a vicious cycle is established in which the inflammatory mediators play a double role by: a) activating pathways leading to neurodegeneration; and b) perpetuating a cascade of neuropathology. Although this hypothesis is based on existing evidence, more studies are required. Intervention studies would provide the needed direct evidence implicating PerioD in the pathogenesis of AD.

In addition, the unique features of PerioD including chronicity, prevalence, association with gram negative bacteria capable of evading the host immune system and their products such as LPS, induction of pro-inflammatory cytokines, and clinical ease of access, may provide a human model to investigate the role and the mechanisms through which peripheral infections and dysbiotic conditions (i.e. gut dysbiosis) contribute to the pathogenesis of AD.

PATHOGENESIS OF AD

Inflammation and pro-inflammatory cytokines

The central tenet of the inflammatory hypothesis is the presence of inflammation in the brain that becomes self-perpetuating and induces neurodegeneration [14–16]. Factors that initiate and maintain inflammation in AD are unknown but potential factors include Aß42 found in senile plaques, P-Tau found in neurofibrillary tangles, or components of the degenerated neurons themselves [17–19]. These factors are able to stimulate glial cells to produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß), interleukin-6 (IL-6) and inflammation associated factors such as CRP [20–22] that not only perpetuate the inflammatory cycle but also are effectors in the pathway of neurodegeneration.

Evidence for a role of pro-inflammatory cytokines in AD comes from studies of clinical specimens and mechanistic-based *in vitro* studies. Immunocytochemistry data has shown that senile plaques immunoreact with antibodies against TNF-α, IL-1ß, IL-6, CRP and complement proteins [23, 24] and that these plaques are associated with reactive astrocytes and activated microglial cells [17]. *In vitro* studies have shown that TNF-α, IL-1ß, IL-6 can stimulate the synthesis of Aß42 and phosphorylation of tau protein [18, 19, 25]. In addition, Aß42 and P-Tau are capable of stimulating the production of TNF-α, IL-1ß, IL-6 by glial cells and Aß42 can activate the complement cascade that can activate and amplify pathways leading to neurodegeneration [25, 26].

In clinical studies, elevation of CRP (an acute phase protein synthesized mainly by the liver during systemic inflammation) and other pro-inflammatory markers were found to be predictive of AD. High levels of CRP were found to increase the risk of AD up to 3 fold [27] and cognitive decline in various populations [28–32]. A nested case-control study of 1,050 subjects derived from the Honolulu-Asia Aging Study showed that higher levels of CRP increased the risk of developing AD in the following 25 years [33]. In addition, CRP was an independent risk factor for delirium. Delirium, an acute psychiatric condition presents mostly in elderly people following surgical interventions and it is characterized by severe cognitive impairment and risk for AD [34, 35].

The literature investigating the pro-inflammatory cytokines as predictors of AD is not as clear. Elevated IL-6 moderately increased the risk of AD

even after adjusting for age, gender, smoking, body mass index, medications and diabetes and correlated with disease severity [31, 32, 36]. Elevated IL-1ß and TNF-α increased the risk of cognitive decline in AD and elderly subjects [7] while subjects with increased production of IL-1 and TNF-α by peripheral blood mononuclear cells were at increased risk of developing AD [37]. Children with Down syndrome have been reported to have a higher risk of developing AD and have higher plasma levels of IL-6, CRP and cell adhesion molecules compared to control children [38]. However, other studies, such as the Longitudinal Aging Study Amsterdam, did not find an association between serum IL-6, CRP and cognitive decline but did find an association with alpha(1)-antichymotrypsin (ACT), an acute-phase protein [39]. The discrepancy in findings is not surprising. In general, the measurement of pro-inflammatory cytokines at a single time point may not be reflective of levels over time or may not reflect levels when challenged by infection. Often IL-6, IL-1ß and TNF-α are operative in several effector pathways and their affect may be due to additive/synergistic roles or by contribution from other factors. In addition, most of the cited studies investigated only selected cytokines, therefore reflecting a limited aspect of inflammation. Support for this later hypothesis comes from a published study in "Nature" that showed that a group of 18 molecules including several inflammatory molecules found in plasma could be used to predict the progression of mild cognitive impairment to AD [40]. The genome-wide association studies showed that several genes encoding proteins of the inflammatory-immune system (PICALM, CLU, CR1, CR2, TREM2, CD33) associated with AD [41–45]. And still other lines of evidence come from clinical studies showing that peripheral infections and inflammations associate and predict cognitive decline and AD. Infectious agents such as cytomegalovirus (Glow, AJ, 2013), Helicobacter pylori [46, 47], spirochetes and herpes simplex virus [48–52] associated with AD pathology and cognitive dysfunction/AD. In addition, peripheral inflammations with significant inflammatory burden such as diabetes, obesity, metabolic syndrome and atherosclerosis also associate with cognitive dysfunction and AD and are now acknowledged risks for AD [35, 53–57].

Cytokine gene polymorphisms and their association with AD have been studied. For a review see (Licastro, 2007). In particular, the presence of a composite genotype characterized by IL-1α-889 and IL-1β+3953 polymorphisms conferred an almost 11-fold increased risk of developing AD [58], presumably due to increased IL-1 levels. It should be noted that the presence of IL-1α-889 and IL-1β+3953 polymorphisms has also been associated with a 7-fold increased risk of periodontitis in non-smokers [59]. These studies suggest that, although AD and PerioD are separate diseases with unique pathogenic bases, their onset, severity and progression may be influenced by common risk factors. Offenbacher [60]in the dental literature and McGeer in the neurological literature [61] suggest that an inflammatory trait may exist which is characterized by an amplified response to an injurious stimulus [60]. This inflammatory trait may increase susceptibility and modify the expression of a disease with an inflammatory etiology. In line with these data, we showed that subjects with periodontal inflammation and having IL-1082 AA/AG genotype tested lower on the cognitive test compared to periodontal subjects with IL-1082 GG genotype or subjects without periodontal inflammation [62]. Subjects with IL-10-1082 GG genotype have increased production of the anti-inflammatory cytokine IL-10 compared to subjects with IL-10-1082 AA/AG genotype. Therefore, this study showed that when a peripheral inflammation is associated with a proinflammatory genotype, it might have significant more effects on the brain.

Additional support for the "inflammatory hypothesis" comes from studies that suggest that anti-inflammatory drugs may slow the onset of AD. The Baltimore and Rotterdam studies showed that a history of anti-inflammatory drug use of at least 2 years duration reduced the risk of AD, suggesting that a reduction in inflammation protects against the onset of dementia [63, 64]. In addition, a meta-analysis supported the beneficial effect of anti-inflammatory drugs in decreasing the risk of AD [65]. However, other studies have not found anti-inflammatory drugs to decrease risk for AD and have offered alternate explanations and they included dosage and type of the drug, APOE status [66, 67] and biological effect. For example, ibuprofen and indomethacin has been reported to lower the Aβ42 (important role in the formation of amyloid plaques) [68] while celecoxib increased its production [69]. Alzheimer's Disease Anti-Inflammatory Prevention Study (ADAPT) investigated the effect of COX2 inhibitors in subjects with Mild Cognitive Impairment (MCI). MCI is a condition in which AD-specific pathology may be already present. The results showed that the COX2 inhibitor rofecoxib failed to decrease the AD

conversion rate. Moreover, it appears that subjects on rofecoxib were 1.46 more likely to convert to AD compared to patients on placebo [70]. Comparable results were also evident in subjects with cognitive impairment [71]. However, when the analysis included only non-symptomatic subjects, naproxen was effective in decreasing the AD risk. Another study investigated the effects of naproxen in 3 groups of subjects with different decline status. Naproxen slowed the cognitive decline in slow declining subjects but actually accelerated the decline in those with fast decline [72, 73]. These dichotomized findings can be explained by the possibility that inflammation can also have beneficial effects on AD pathology. This hypothesis is also supported by animal studies. For example, when Aβ-antibodies were administered to mice, there was a decrease in Aβ pathology, and increase in cognitive performance, and when the inflammatory response provoked by this procedure was attenuated, the effect on the amyloid pathology was also attenuated [73]. Inhibiting TNF-α signaling pathway in a mouse model of amyloid deposition lead to more amyloid accumulation and tau pathology. In addition, transgenic mice with enhanced IL-1 (an important proinflammatory cytokine) expression showed reduced Aβ plaques (Shaftel, SS, 2007). These studies question a strait-forward role of inflammation in AD and raise the idea of a dichotomized role of inflammation [72]. It appears that inflammation has a deleterious effect early in the AD process but it may even be beneficial at later stages given a competent immune system. Collectively, these studies do not refute the "inflammatory hypothesis" but suggest that more studies on the role and mechanisms of inflammation and anti-inflammatory drugs in the pathogenesis of AD are needed.

Relatively large pro-inflammatory molecules such as TNF-α, IL-1β and IL-6 have limited access to the brain. Nonetheless, evidence exists that these molecules reach the brain by at least two mechanisms: a) systemic circulation and b) neural pathways (reviews by Banks and Quan and Banks) [74, 75]. Cytokines within systemic circulation may affect blood-brain barrier (BBB) permeability, may bind to areas of the brain that lack a BBB such as circumventricular organs [76, 77], may cross through fenestrated capillaries of the BBB or may use cytokine-specific transporters [77]. Cytokines may also activate brain endothelial and perivascular cells [78] to induce production of other signaling molecules such as nitric oxide (NO), prostanoids or other cytokines that in turn stimulate glial cells [75, 79, 80]. Thus, peripheral molecules may increase the existing brain cytokine pool concentration by addition or by glial stimulation. If the glial cells are already primed (activated), as is likely to occur with increasing age or display an hyper-inflammatory phenotype, stimulation will result in amplified responses with considerable inflammatory molecule production [81–83]. Peripheral cytokines may also impact the brain pro-inflammatory cytokine pool through neuronal pathways [84]. This mechanism implies that peripheral cytokines stimulate afferent fibers of peripheral nerves leading to increased brain cytokines. The significance of this mechanism is that signaling cytokines may be only needed to be elevated locally and not systemically [85]. Although, this mechanism has been mostly described for the vagus nerve [86], nerves enervating the oral cavity such as the glossopharyngeal and trigeminal nerves have also been proposed [87].

Bacterial products may also increase brain cytokine levels. Lipopolysaccharide (LPS), a component of Gram negative cell walls and a potent pathogen-associated molecular pattern for the innate immune response, is capable of increasing peripheral cytokine concentrations and up-regulating CD14 receptors throughout the brain [88]. There, CD14 can be activated by existing Aβ protein or LPS derived from invasive bacteria increasing further the brain cytokines. Consistent with this mechanism, peripheral administration of LPS into APPswe transgenic mice increased the accumulation of amyloid precursor protein (APP) and Aβ [89]. Another possibility is that peripheral LPS may increase the permeability of the BBB [90] allowing the passage of at least some molecules, cells and possible bacteria into the brain.

Peripheral infection and AD

Linked with the inflammatory hypothesis is the pathogen hypothesis that suggests some pathogens act as triggers or co-factors in the etiology and pathogenesis of AD [91]. This hypothesis has been recently strengthen by Kumar et al. [92] showing that in an animal model Salmonella Typhimurium bacterial infection lead to β-amyloid deposition. Clinical studies also support the role of infections in AD pathogenesis. A prospective study showed impaired cognitive function in AD patients for at least two months after the resolution of a systemic infection [7]. In addition, peripheral infections were reported to increase the risk of delirium in patients with AD [93]

and in a twin study, a history of past severe peripheral infections accelerated the onset of AD [8]. The amount of evidence showing the role of microbes in the pathogenesis of AD is high. Therefore, editorial co-authors by tens of investigators called for more research including intervention studies [94].

Several bacterial species have been implicated in the pathogenesis of AD including *Chlamydia pneumoniae*, *Helicobacter pylori* and spirochetes, although conflicting evidence exists for some bacteria. One post-mortem study reported *C. pneumoniae* present in 17 of 19 samples from individuals with AD, but only present in 1 of 18 samples from non-AD, age matched controls [95]. However, another study did not replicate this finding [96]. Higher serum IgG antibody against *H. pylori* has been reported in AD subjects compared to controls [97]. Spirochetes were reported present in blood, CSF and brain samples from 14 AD cases but were absent in 13 controls lacking Alzheimer's symptoms [98, 99]. Although not all species of spirochetes were characterized, at least some of the spirochetes were *Borrelia burgdorferi* [100]. These finding were consistent with MacDonald's findings that cultivated *B. burgdorferi* from brain samples from AD patients [101]. In addition, *B. burgdorferi* specific antigens were co-localized with Aβ deposits and glial and neuronal cells exposed to *B. burgdorferi* were able to produce AβPP and hyperphosphorylated tau proteins [102], suggesting that *B. burgdorferi* is able to induce AD specific pathology. Of interest, spirochetes from the oral cavity have been reported in brain samples from AD patients by Riviere using molecular and immunological methods [103]. These results suggest, as did Miklossy that spirochetes within brain tissue may originate from diverse areas including the oral cavity [98]. Recent studies support these ideas by showing that P gingivalis was found in subjects with AD but not in those without AD [104].

PERIODONTAL DISEASE

PerioDs are a heterogeneous group of diseases that affect the supporting structures of the teeth. The most common forms of PerioD are associated with bacteria in the dental plaque and they are dental plaque-induced gingival diseases or gingivitis, chronic and aggressive periodontitis. Gingivitis is an inflammatory, reversible condition limited to the gingiva characterized by erythema, edema, bleeding and gingival enlargement. Gingivitis is prevalent in both children and adults ranging from 30% to 90% in children and 40–50% in adults [105]. Chronic and aggressive periodontitis (in this review they will be referred as periodontitis) are destructive and irreversible forms of PerioD in which the inflammation extends from the gingiva to the tooth's attachment apparatus including the bone. Clinically, periodontitis presents similar features to that of gingivitis but in addition, there is soft connective tissue and often bone loss creating deep, ulcerated pockets (groove between the tooth and its supporting tissue) around the teeth that ultimately lead to tooth loss. It was estimated that in subjects with periodontitis the surface of epithelium lining the pockets ranged from 8 to 20cm^2 suggesting large areas of possible concealed, ulcerated surfaces [106]. Approximately 45% of the dentate U.S. adults representing 64.7 million people have periodontitis, with 8.9% having severe periodontitis [107]. In addition to adults, 2-3% of children have chronic periodontitis and another 0.2–2% have a severe form called aggressive periodontitis [108]. Aggressive periodontitis affects young people and in specific populations such as Down syndrome is quite prevalent [109].

Pathogenesis of PerioD

A balance between bacteria populating the dental biofilm and host immune response [59] maintains the health of periodontal tissue. In gingivitis, the host innate and adaptive immune systems are able to control the bacterial biofilm and its effects. In periodontitis however, the balance between bacteria and host response is disturbed, resulting in an uncontrolled inflammation characterized by the production of high levels of inflammatory mediators such as IL-1, IL-6, 17 and TNF-α, and low levels of anti-inflammatory molecules such as IL-10 [110, 111]. These molecules act in concert to amplify the inflammatory reaction and activate the effector mechanisms responsible for tissue destruction in PerioD (Fig. 1). Metalloproteinases are also activated and collagen synthesis is inhibited. Cells including T and B cells are stimulated to express receptor activator of nuclear factor κB ligand (RANKL) [112] a significant factor in osteoclast activation. RANKL binds to receptor activator of nuclear factor κB (RANK) found on osteoclasts and signals them to proliferate, differentiate and become activated. Activated osteoclasts then resorb the alveolar bone that ultimately leads to tooth loss. A vicious cycle is developed in which the resulting inflammation and products of

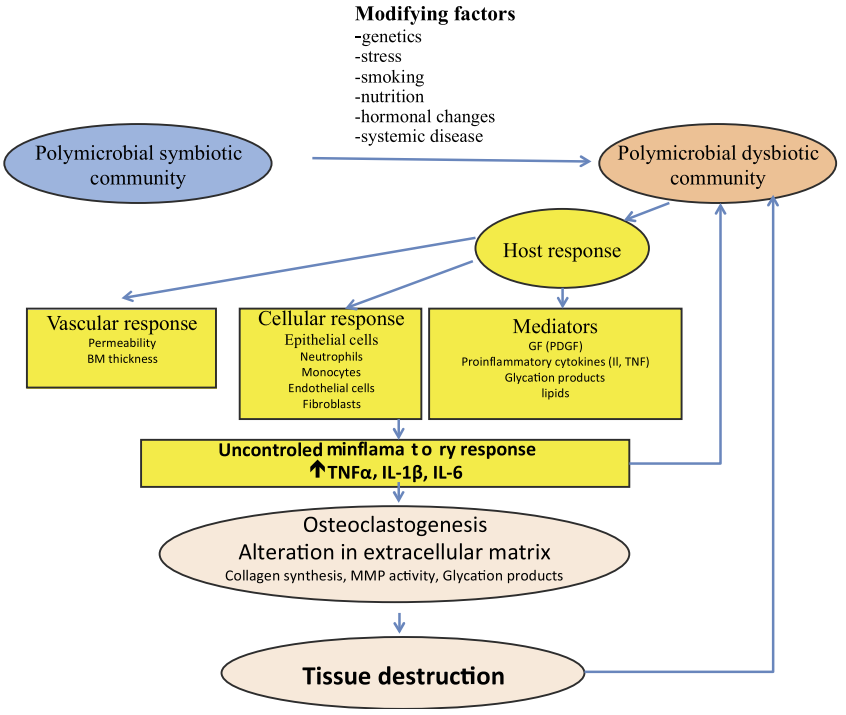

Fig. 1. Pathogenesis of periodontitis. In disease susceptible individuals, the balance between symbiotic bacteria and host response is disturbed leading to a dysbiotic microbial community. The microbial dysbiosis activates host innate and adaptive immune responses resulting in an enhanced local and systemic release of inflammatory mediators such as IL-1β, IL-6 and TNF-α. In turn activation of the host response results in osteoclast activation, decreased collagen synthesis, and increased expression and activity of metalloproteinases leading to tissue destruction. A vicious cycle ensues where inflammation and products of tissue destruction contributes to further dysbiosis and inflammatory responses. Risk factors for periodontitis include genetics, inadequate oral hygiene, stress, smoking, nutrition, hormonal changes, and systemic diseases, such as diabetes mellitus.

tissue destruction contributes to further bacterial dysbiosis and inflammatory responses [113, 114]. These findings show that inflammatory reactions mounted by the host impacts the occurrence and the expression of PerioD.

Periodontal bacteria

PerioD is a chronic, peripheral, polybacterial inflammatory disease. Periodontal bacteria exist within a complex ecosystem called dental biofilm forming on the tooth surface. In addition to microorganisms and their components (endotoxin, virulence factors), dental biofilm is composed of other proteinecious and nonproteinecious materials providing a system in which periodontal bacteria growth is favored and protected from the host defense mechanisms or antibacterial drugs [115].

Periodontal bacteria are required for the initiation, maintenance and progression of periodontal diseases. Among them Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), and members of the red and orange clusters such as Tenerella forsythus (T. forsythus), Porphiromonas gingivalis (P. gingivalis) Treponema denticola (T. denticola) and Fusobacterium nucleatum, Prevotella intermedia, Prevotella nigrescens, Parvimonas micra, Streptococcus constellatus, Eubacterium nodatum, Campylobacter showae, Campylobacter gracilis, and Campylobacter rectus are considered important periodontal pathogens [116]. A. actinomycetemcomitans is known for its role in localized aggressive periodontitis, and A. actinomycetemcomitans and P. gingivalis in generalized aggressive periodontitis. With some variation, the red and orange complex (Haffajee and Socransky, 1997) are more prevalent at diseased sites compared to healthy sites of the periodontitis subjects, at progressive sites compared to the non-progressive ones, and at healthy sites of periodontitis subjects compared to the healthy sites of the healthy subjects [117, 118]. In addition to above bacteria other bacterial flora have been found to contribute to periodontal disease pathogenesis such as Porphyromonas endodontalis, Treponema lecithinolyticum,

Treponema medium, Filifactor alocis, and S. sputigena. Other bacteria are associated with periodontal health. For example, Veillonella parvula, Actinomyces sp., or the combination of Streptococcus oralis, Streptococcus mitis and Streptococcus intermedius are considered beneficial and may be protective from the periodontal disease [review [117, 119].

The development of new molecular techniques led to novel concepts related to the pathogenesis of periodontal disease. According to this model, periodontal disease results from the induction of a microbial dysbiotic community. The oral microbiota constitute one of the most diverse and abundant ecosystems in our body. Approximately 1000 bacterial species colonize the oral cavity with any particular individual holding more than 200 species. Up to 700 species colonize the subgingival biofilm (under the gingival line), most are anaerobic, and implicated in periodontal disease [120, 121]. The diversity, the abundance, as well as the gene transcription, protein/virulence factors expression, metabolic make-up of the specific bacteria in the biofilm are molded by a constant, multidirectional communication between bacteria, environment, host genetics and its immune system [122–125]. Likewise, the host immune system is constantly modulated by the bacterial activities. In periodontal health the interrelationships between bacterial challenge and the host immune response are balanced [122] constituting a symbiotic microbial community (Fig. 1). However, when predisposing conditions arise in disease susceptible individual, keystone pathogens (bacteria with significant effects despite their relative low abundance) such as P. gingivalis and perhaps A. actinomycetemcomitans and T. denticola evade the host immune system and initiate events leading to microbial shifts called dysbiosis. Within dysbiosis, the commensal flora become pathobionts with active roles in activating host immune inflammatory reactions that led to periodontal tissue destruction. The resulting inflammation maintains and perpetuates the microbial dysbiosis and thus a vicious cycle is formed. Accessory pathogens can aid in keystone and/or pathobionts-induced pathogenesis [113, 114, 125–127]. The significance of this pathogenic concept is multifold. It explains the results showing that healthy sites also harbor "disease inducing keystone" bacteria. It shows that the composition of the whole microbial population is health or disease-relevant and certainly the immune system plays [113] a major role. In addition, as shown previously, treatment of periodontal disease results not only on reduced bacterial counts [128, 129] but also on a shift in bacterial composition towards healthy flora. Using metagenomics, transcriptomes and metabolomics would add additional understanding of the biology and how these bacteria can impact the host. Studying bacterial flora in the context of the immune system would provide a better picture.

In addition to periodontal bacteria, there is evidence to suggest a role for human herpesvirus species particularly Epstein–Barr virus (EBV) and type 1 human cytomegalovirus (HCMV) in the pathogenesis of periodontitis [130]. These findings however are relatively new and more research is needed to investigate their contribution to microbial dysbiosis and periodontitis and systemic diseases [131].

Host response in PerioD

Inflammatory chemokines such as IL-8, Monocyte chemotactic protein-1 (MCP-1) and cytokines such as IL-1β, IL-6, IL-17, IL-23, G-CSF and TNF-α have a prominent role in the pathogenesis of PerioD [132–134]. They are elevated within the diseased periodontal tissues, gingival crevicular fluid (GCF) and in plasma suggesting their chronic production.

Local host response in periodontitis: GCF is an exudate expressed in the gingival sulcus and is considered a "window to PerioD" [135]. In addition to molecules derived from serum, GCF is also composed of substances derived from interstitial tissue and cells [135]. Up to 94% of inflamed periodontal sites have elevated levels of IL-1β in the GCF collected from those sites [136]. Progressive disease has higher levels of IL-1β in their GCF than nonprogressive disease [137, 138] and treatment of PerioD decreases IL-1β values [139]. Within subjects with periodontitis, healthy sites had higher values of IL-1β than healthy sites from subjects with mild periodontitis [139] suggesting that GCF production of inflammatory mediators may be subject specific. This later hypothesis is supported by studies showing that periodontal subjects with specific polymorphisms in the IL-1 gene produce 2.5 more IL-1β in shallow pockets than subjects having periodontitis but without these polymorphisms [140]. IL-6, TNF-α, IL-17, IL-8, IFN-γ and MCP-1 have also been found to be increased in GCF of patients with PerioD [141–143].

Systemic host response in periodontitis: PerioD is a localized disease but when present in severe forms may induce a systemic inflammation demonstrated by the elevation of inflammatory markers in the blood such as C-Reactive Protein (CRP) and various cytokines. CRP is an acute-phase response

protein that is known to increase up to 1000 folds in acute inflammatory diseases [144]. CRP also increases in chronic inflammatory conditions. In rheumatoid arthritis, CRP values may well exceed 100 mg/L while in chronic protracted conditions such as those caused by *H. pylori* or *C. pneumoniae* may be only slightly to moderate elevated (median 0.3–7.99 mg/L) [145]. However, the attention on CRP levels stems from its predictive value of cardiovascular diseases. The US Centers for Disease Control and Prevention and the American Heart Association [146] defined subjects at average risk for CVD if CRP is 1.0–3.0 mg/L, and high risk if CRP is >3.0 mg/L. Subjects with periodontal disease have been found to have elevated CRP values compared to healthy controls [147–149] particularly when they had severe disease [147] with means exceeding 3 mg/L [150]. For example, data derived from National Health and Nutrition Examination Survey III showed that subjects with extensive PerioD were more likely to have high levels of CRP [> 10 mg/L] than subjects without PerioD [151]. In fact, 12.5% of subjects with extensive PerioD had high CRP levels compared to 6% subjects without PerioD. These high levels of CRP denote a significant inflammatory state, are in the range that is considered risk for cardiovascular disease [152] and are a consistent finding (meta-analysis) [153]. Treatment of PerioD resulted in the decrease of CRP levels and other atherosclerotic markers particularly in those responsive to periodontal treatment and with additional co-morbidities (CVD/diabetes) [154, 155] supporting the notion that the elevated CRP was PerioD related. The presence of systemic IL-1β, IL-6, IL-17 and TNF-α was also examined although these studies are limited. However, they suggest that subjects with periodontitis have higher systemic IL-6 and IL-17, IL-21 compared to controls [156–158]. Plasma IL-6 and TNF-α may decrease following periodontal treatment suggesting that these markers reflect periodontal infections [159]. However, plasma level of IL-1 and TNF-α appear to depend on the severity of injury [160] and therefore their levels may be more difficult to detect if PerioD is not severe or extensive enough.

PerioD as a risk factor for other systemic diseases: In addition to periodontal pathology, periodontopatic bacteria are also capable of causing systemic pathology. Examples of these pathologies are endocarditis [161], brain and lung abscesses and pulmonary disease [162]. In the first instances periodontal bacteria gained access to the systemic circulation and metastasized at distant sites, however, in pulmonary disease periodontal bacteria reached the pulmonary tree by aspiration [163, 164]. Other systemic diseases associated with PerioDs are diabetes, low weight birth, cardiovascular and renal diseases [13, 165–171]. In these instances two mechanisms of action may be involved: 1) direct bacterial/bacterial products action at the site of pathology through bacteremia, endotoxemia and virulence factor release in the circulation and 2) host response mechanisms to periodontal bacteria implicating the inflammatory mediators that are released systemically. Bacterial mimicry is still another possible mechanism [172].

Bacteremia derived from oral cavity is a frequent occurrence during treatment or even examination and depends on the procedures performed and the presence of gingivitis/periodontitis or severity of periodontitis [173]. Even daily procedures such as flossing, brushing and mastication may induce bacteremia with a comparable prevalence to the one induced by dental procedures [174] and the frequent nature of these procedures may lead to significant bacterial exposures [175]. Metastasizing at distant sites periodontal bacteria is capable of inducing pathology. For example, *A. actinomycetemcomitans, P. gingivalis* and T. denticola were recovered in atherosclerotic plaques [176, 177] and *P. Gingivalis* induced the expression of adhesion molecules and proinflammatory cytokines in aortic tissue and accelerated atherosclerosis in an animal model (apolipoprotein E-deficient mice) [178]. In another animal model (BALB/C mice), infection with *C. rectus* induced decreased expression of Insulin Growth Factor 2 (IGF2) mRNA by epigenetic modification of the Igf2 gene [179] and induced placental structural changes. These studies provide evidence for the diversity of molecular effects that periodontal bacteria are capable of inducing and possible mechanisms of actions for periodontal bacteria.

Endotoxemia may occur upon professional and nonprofessional dental manipulations and may be accompanied by elevation in TNF-α, IL-6, CRP [159, 180] and possible mild fever [181] suggesting an association with a systemic acute phase response.

ASSOCIATION BETWEEN PERIODONTAL DISEASE AND PROGRESSION OF AD

Despite major advances, the mechanisms involved in the pathogenesis of AD are not understood. However, inflammation is believed to play a significant role [182], and as such, processes capable of increas-

ing the brain inflammatory state may contribute to the progression of AD. PerioD is a chronic inflammatory disease resulting in years of significant bacterial and inflammatory local and systemic exposure. We propose the hypothesis PerioD may enhance the inflammation in the brain and contributes to the progression of AD (Fig. 2). We propose that two mechanisms may be involved in the PerioD-induced progression of AD: 1) inflammatory and 2) bacterial mechanisms. The first mechanism implies that PerioD-derived inflammatory molecules increase brain inflammation. As described in the previous paragraph, the interaction between periodontal bacteria and host response results in locally increased production of inflammatory molecules including IL-1ß, IL-6, IL8, TNF-α, IL-17, IL-18 and CRP. In severe or extensive PerioD these proinflammatory molecules may also induce systemic inflammation and therefore may access the brain via systemic circulation. Proinflammatory molecules derived locally from periodontal tissue may also stimulate trigeminal nerve fibers, leading to increased brain cytokines [87]. These cytokines may act on the already primed glial cells resulting in an amplified reaction and possible progression of AD. A test of this hypothesis would entail examining whether PerioD affects the progression of AD throughout its clinical course.

The second mechanism by which PerioD could contribute to brain inflammation is through bacteria and/or bacterial products. Several bacteria including oral ones are hypothesized to be implicated [52, 183–186] in the pathogenesis of AD. Among periodontal bacteria, species such as *A. actinomycetemcomitans*, *P. gingivalis, T. denticola* and *F. nucleatum* are capable of invading the brain, changing the cytokine milieu and possibly contributing to existing pathological mechanisms. For example, *Treponema* species including *T. denticola* were detected in 14/16 AD and 4/18 non-AD brains. In addition, AD specimens also had more *Treponema* species than controls [103]. Similarly, *P. gingivalis* was found in subjects with AD but not in those without AD [104]. In an animal model of oral infections *T. denticola* was detected postmortem in the brain [187]. These findings are not surprising since *T. denticola* is from the same class as *Treponema pallidum*, which is also

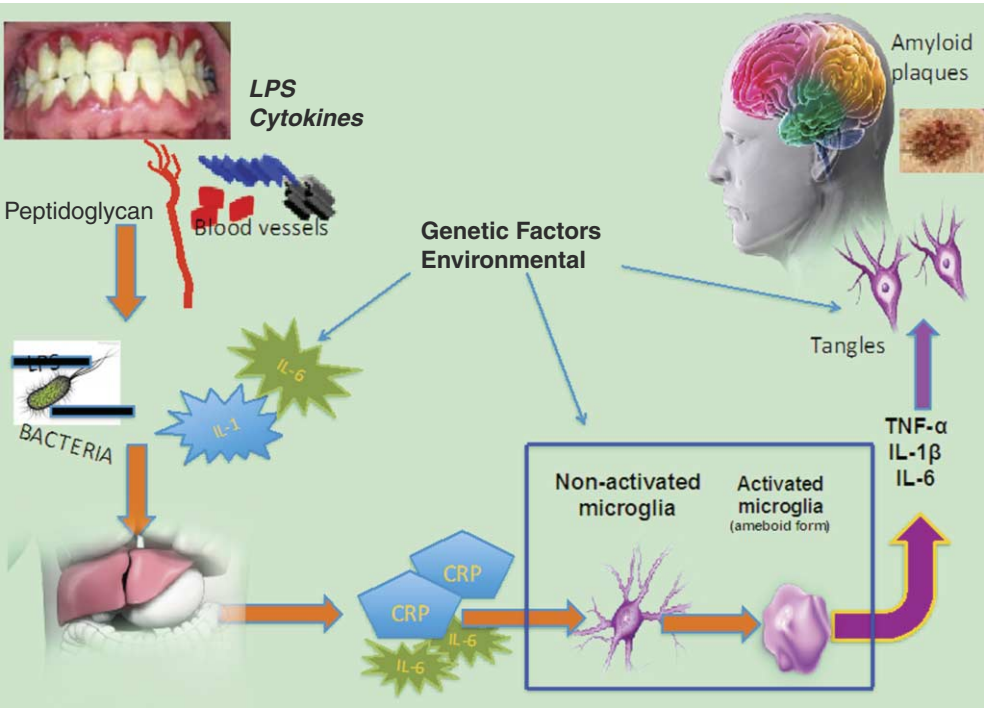

Fig. 2. Model for PerioD-induced progression of AD. The central theme of AD pathogenesis is brain inflammation as illustrated by the activated glial cell that produces high levels of inflammatory molecules such as Il-1β, Il-6, TNF-α and CRP. PerioD may affect the initiation, progression of AD by directly (bacterial invasion) or indirectly (LPS, cytokines, CRP) increase brain inflammation via neuronal or systemic pathways. These molecules would further amplify the inflammatory signal by activating the already primed glial cells and increase production of molecules such as β amyloid peptide, hyperphosphorylated tau proteins and ultimately activate pathways leading to degeneration. Genetic and environmental factors can modulate each step of this process.

known to invade the brain, induce chronic inflammation, cortical atrophy and amyloid deposition in subjects with syphilis. Reports of brain abscess in which oral bacteria such as *A. actinomycetemcomitans*, *F. nucleatum* and possible *P. gingivalis* are implicated attest to their capabilities to invade the brain and induce pathology.

Once in the brain, periodontal bacteria that are rich in LPS or their products are capable of stimulating cytokine production. For example, heat-killed *P. gingivalis* administered through a subcutaneous chamber to mice with induced experimental autoimmune encephalomyelitis aggravated the disease compared to controls [188] possibly through an inflammatory mechanism as *P. gingivalis*-derived LPS stimulated NO and prostaglandin E2 (PGE2) in rat glial cells [188, 189]. Since *P. gingivalis* LPS stimulates the human cells through CD14 and toll-like receptors (TLR-2 and 4), it has been suggested that perhaps brain-induced inflammation induced by *P. gingivalis* -derived LPS may be mediated by these receptors [190]. Additional evidence comes from Offenbacher's studies [191]. In a subcutaneous chamber infection model, challenge of embryonic mice with *C. rectus* resulted in hippocampal morphological changes in pups including cytoplasmic vacuoles and cellular debris suggestive of cellular damage. Molecularly, the fetal brain of challenged embryos had about twice more TNF-α and IFN-γ m-RNA compared to nonchallenged embryos [191]. In the brain cytokines and LPS are the primary candidates as they were found to consistently stimulate amyloid synthesis and induce cognitive impairment [6, 82, 192]. Our own study showed that in normal subjects, measures of history of periodontal disease associated with amyloid accumulation in the brain [193]. It has been hypothesized that Aβ may be anti-microbial [92, 194] that when is unregulated could cause brain damage. PerioD can certainly fit within this model. Brain inflammation and bacterial products can also affect tau protein hyperphosphorylation [102]. We have shown that subjects with periodontal inflammation have higher levels of p-tau and t-tau proteins in their CSF [195], a finding consistent with the role of infection/inflammation in tau pathology and brain damage.

The mechanism by which periodontal bacteria have access to the brain is not known. However, the mechanisms described for other bacteria such as access via systemic circulation is possible. Bacteremia of oral origin occurs quite frequently during dental and nondental manipulations. Other ways bacteria may reach the brain is via peripheral nerves. Riviere's studies showed that spirochete species were detected in the trigeminal ganglia and pons suggesting the ability of oral spirochetes to invade CNS via peripheral nerves [103]. However, the mere presence of periodontal bacteria in the systemic circulation or in peripheral nerve fibers territory does not imply access to the brain. Perhaps additional cofactors are needed such as age, the presence of inflammatory cytokines or other infections [90, 196].

Other mechanisms have been proposed for periodontal bacteria induced systemic pathology: reduced masticatory abilities due to periodontal disease may result in dietary deficiencies and increased stress response may lead to increased Aβ [197]. It is also possible that the inflammatory/infectious burden could directly affect synaptic and neuronal dysfunction [198].

Clinical data from our studies [193, 195, 199, 200] and others have provided evidence of a link between periodontal disease, AD/cognition and AD-specific pathology. Cross-sectional and longitudinal studies have reported that measures of periodontal disease were associated with cognitive impairment, cognitive decline, dementia and AD at least in some populations [4, 201–212]. The odd ratios (OR) varied from slight [213] to strong [200] and this is not surprising considering the variety of exposure indexes, study designs, populations and outcomes. For example, three longitudinal studies showed that pocket depth, periodontal inflammation and diagnosed severe periodontal disease predicted cognitive decline with mild to moderate strength [HR = 1.05 (95%CI: 1.01–1.10); OR = 1.57 (95%CI: 1.01–2.45); RR = 2.2 (95%Cl: 1.1–4.5)] [211, 213, 214]. The presence of periodontal disease also impacted the cognitive decline in subjects with AD. In fact, Ide et al. [212] showed that the cognitive decline in AD subjects with periodontal disease was six fold higher than in AD subjects without periodontal disease. Moreover, limited data suggested that the treatment of oral disease might have a beneficial effect on cognition [215, 216]. Other studies did not support this link particularly in some populations [202, 217]. Stronger relationships were found when periodontal disease was defined by immunological parameters [199, 218–220]. In a nested case-control study Sparks [218] showed that subjects that converted to AD had higher IgG antibodies to periodontal bacteria 10 years before their conversion. Perhaps, immunological markers for PerioD representing microbial exposure and the host immune responses to the micro-

bial challenge are stronger predictors than just the clinical markers. A significant challenge in performing these studies arise from the intense work required evaluating periodontal disease. Therefore, most studies used tooth loss as an exposure and the results were consistent with significant associations with OR between 1.05 and 2.38. These results were confirmed by a meta-analysis [221]. Indeed, tooth loss is the ultimate outcome of periodontal disease and its use in the majority of cohort studies as a proxy for PerioD is derived from its convenient assessment – it can be obtained by subject report and can easily be added to an existing study. Therefore, studies aimed at deciphering the role of PerioD in AD pathogenesis must be designed specifically for this purpose.

In addition to PerioD, caries (tooth decays) and endodontic complications are significant causes of tooth loss and the possibility that these oral infections may contribute to AD pathogenesis cannot be excluded.

CONCLUDING REMARKS

AD prognosis has not changed significantly as understanding the mechanisms involved in disease initiation and its progression is still lacking. We propose that PerioD with significant bacterial and inflammatory burden may enhance the inflammation in the brain and contributes to the initiation/progression of AD through effects on AD specific pathology. Alzheimer's Association [222] concluded that several inflammatory conditions such as diabetes, obesity, and hyperlipidemia should be considered risk factors for AD and therefore, interventions to reduce their prevalence should be at the forefront of AD prevention. Periodontal disease is an inflammatory disease and significant evidence exists showing its role as a potential risk for AD. Therefore, acknowledging that periodontal disease may also be a risk factor for AD [223] is highly important as its acknowledgement would encourage research into "out of the box" thinking, create widespread collaborations between medical and dental professionals on the Alzheimer's disease front and provide a means to alter AD course. Periodontal disease is highly prevalent in the general population (in USA, 45% of dentate adults have PerioD representing 64.7million people). Moreover, specific forms of periodontal diseases begin early in life, are prevalent in some populations such as Down syndrome [109, 224] and their contribution to AD could be etiological. Therefore, even if periodontal disease has only a low to moderate effect, preventing or treating it could prevent a significant number of AD cases and therefore deserves unequivocal consideration.

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Putative Association of Periodontitis with Alzheimer's Disease

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Abstract. The mechanisms of disease processes resulting in dementia of which, Alzheimer's disease (AD) is a common example, remain elusive. To this end, a number of theories as plausible explanations have been suggested. Of these, the microbial, peripheral infection theory of Hunter and Miller (1900s) and Naguchi and Moore (1913) is the earliest proposal to explain possible causation of AD. Periodontal disease is a polymicrobial inflammatory disease reported to associate with AD via periodontal bacteria/bacteraemia, systemic inflammation, blood-brain barrier erosion, intra-cerebral inflammation and tissue injury, this chapter describes the original finding of four out of 10 confirmed AD brains with incidental infection of *Porphyromonas gingivalis* [*P. gingivalis*] outer membrane component lipopolysaccharide. A follow-on study examined the possibility of *P. gingivalis* translocation from the gingivae to the brain in the orally infected ($n = 12$), apoliporprotein E knockout ($ApoE^{-/-}$) mouse model at 12 and 24 weeks of monoinfections. Sensitive bacterial molecular speciation techniques confirmed the invasion of *P. gingivalis* into the brain at 12 weeks ($p = 0.006$), and at 24 weeks of infection ($p = 0.0001$). Immunolabeling using antibodies against complement proteins demonstrated the innate immune system activation via C3 fragmentation and its subsequent opsonisation onto vulnerable pyramidal neurons ($p = 0.032$) in the hippocampus as ongoing bystander injury. These studies confirm the initiation of an infection mediated inflammasome assembly with implications for remote body organ inflammatory pathologies from periodontitis to dementia.

Keywords: Apolipoprotein E knock out mouse model, Alzheimer's disease, periodontal bacteria, periodontitis

INTRODUCTION

Dementia is diagnosed in the presence of global cognitive decline sufficient to interfere with everyday activities and may involve progressive decline in memory, concentration, reasoning and behaviour. It is a feature of many neurodegenerative diseases including Alzheimer's disease (AD), Vascular Dementia, Frontotemporal dementia, and Dementia with Lewy Bodies. Of these AD is the most common type of dementia with estimated prevalence of 10–30% after 85 years age.

Alzheimers disease

There are two main forms of AD. The genetic form accounts for only a small proportion (2–5%) of the population with expression of disease at an earlier age [1]. These individuals show mutations in presenilin 1 and 2 and/or the amyloid precursor protein (APP) gene [1] and apolipoprotein E gene allele 4 (APOE $\varepsilon 4$) [2, 3]. The other form of AD, which expresses much later in life is appropriately referred to as the late-onset AD or LOAD. The aetiology of LOAD is not known but is responsible for 95–98% of all cases that go on to developing the disease. There are

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numerous susceptibility genes implicated to LOAD and the top ten are listed in the www.alzgene.org public domain. The Apo ε2/3/4, BIN 1 (bridging integrator 1), CLU (Clusterin), ABCA7 (ATP-binding cassette, subfamily A (ABC1), member 7), CR1 (complement receptor 1) are listed as the top 5 (www.alzgene.org) genes. Of these, the Apoε4 allelic variant is now accepted as an established risk factor for AD in general and particularly in LOAD in which, 50% of the demented subjects appear to have inherited at least one copy. From the other candidate susceptibility genes clusterin and CR1 [4, 5], are related to the innate immune system which combat infections. Both forms of AD are characterised by the presence of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) representing the classical diagnostic hallmark proteins [6]. Inflammation is recognised as an element of AD pathology in response to the Aβ plaque inclusion body associated inflammasome [7, 8] rather than as a result of intracerebral bacterial infections independent of hallmark proteins [8], and/or their virulence factors accessing the demented brains [9–14].

What is a dental-biofilm

The mouth is a natural semi-aquatic system with mucosal and mineralized tissues all in one cavity harboring a biofilm. Biofilms are a community of complex, 3-dimensional arrangement of multispecies of microorganisms [www.homd.org]. Briefly, bacterial colonization occurs on oral surfaces by means of adhesion molecules and acquisition of extracellular polysaccharide matrices derived from organic and inorganic components of bacteria and host proteins (saliva and gingival crevicular fluid). The human dental biofilm is initiated by Gram-positive *Streptococcus* species of bacteria and the biofilm structure, microbiology, and pathophysiology is fully described elsewhere [15]. The rate at which the biofilm progresses varies among individuals and is influenced by oral hygiene, dietary composition, salivary flow rates and the immune defenses. The dental biofilm above the gingival margin accumulates on areas of the tooth surfaces where access for mechanical cleansing is difficult. The dental biofilm undergoes maturation, and progress from gingivitis to subgingival bacterial complexes as those seen in chronic periodontitis [15–17]. The human periodontal subgingival microbiome contains approximately 400 different bacterial species [16, 17], predominantly of the Gram negative phylotypes in which *P. gingivalis* is one bacterium dwelling synergistically with others [18]. This is due to the compatibility of *P. gingivalis* with the quorum sensing molecules (oligopeptides), secreted by other bacteria [19, 20]. It is generally accepted that through quorum sensing, bacteria can determine their population density, type of organisms that co-inhabit the same niche and respond by switching on/off their genes accordingly [21]. *P. gingivalis* demonstrates numerous strategies for colonising and then adapting to and surviving in highly inflammophillic environment [22–26]. The more virulent stains of *P. gingivalis*, for example FDC 381 [27] is fimbriated. This virulence factor allows *P. gingivalis* to adhere and spread to distant organs and once there, can out-compete the inflammophobic flora to establish its colonisation [22–25, 28, 29].

P. gingivalis the chronic periodontal bacterium

P. *gingivalis* has been proposed as the keystone periodontal pathogen in maintaining the periodontal disease associated inflammophillic microbiota [22, 23]. The subversive armoury of *P. gingivalis* (enzymes, proteins, and end-product of their metabolism active against several host proteins) and its strategies to subvert host defences not only provides nutritional sustenance from sustainable inflammatory milieu but also allows for its continued survival in potentially highly toxic niches [24]. Such niches include several systemic diseases: cardiovascular disease, atherosclerosis; with the net effect of increasing vessel wall inflammation, atherosclerotic lesion formation [27, 30, 31]; and AD where these factors will contribute to cognitive decline by enhanced assembly of the Aβ inflammasome associated inflammation [8]. An exhaustive review of virulence factors of *P. gingivalis* was published in 1999 [28] and then specifically related to AD in 2015 [26]. Recent molecular studies show that *P. gingivalis* is a quantitatively minor constituent of human periodontitis-associated subgingival biofilms but its presence enhances other microbial virulence factors synergistically towards dysbiotic state [25, 32].

In Velsko et al. [27] and Poole et al. [33] studies the inflammophillic microbe, *P. gingivalis* strain FDC 381 (Fig. 1) was delivered to the gingivae which led to its subsequent colonization of the subgingival areas and subsequently disseminated to systemic channels and the brain (Fig. 2) confirming a haematogenous path of translocation.

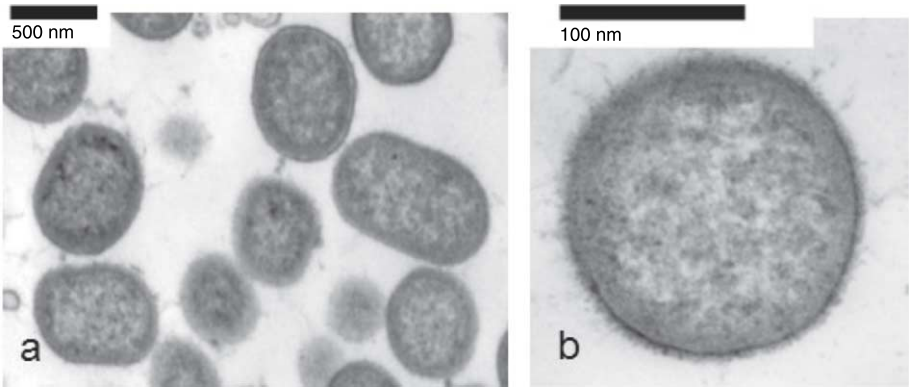

Fig. 1. Transmission electron microscope micrograph of *P. gingivalis* strain FDC381. a) Many bacterial cells to show its rod-shape, bar = 500 nm and b) higher magnification to show fimbriae on the surface membrane, bar = 100 nm.

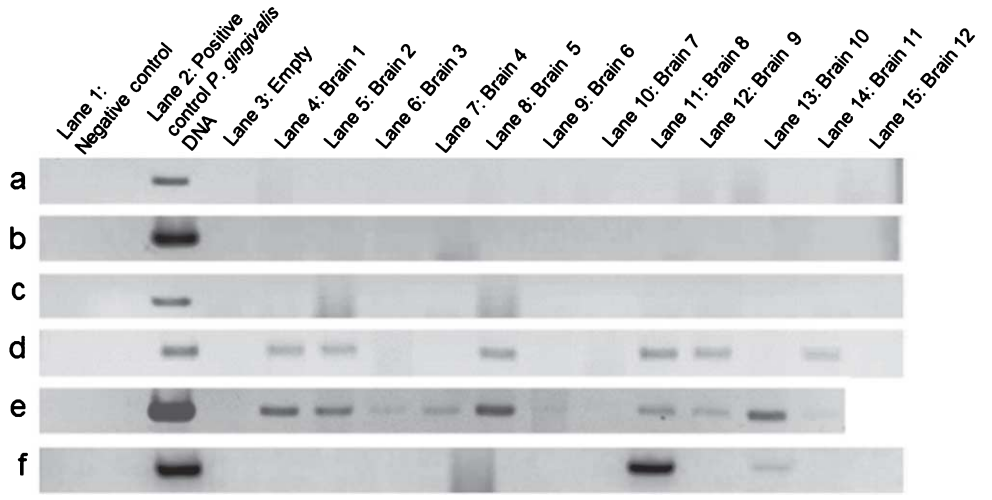

Fig. 2. Taken from Poole et al. [33]. Molecular identification of *P. gingivalis* in ApoE$^{-/-}$ mice brain tissue using specific primers. Panels a and b) mono sham-infected group 12 and 24 weeks, c) polymicrobial sham-infected group 24 weeks, d) Mono-infection with *P. gingivalis* at 12 weeks, e) Monoinfection with *P. gingivalis* at 24 weeks, f) Polymicrobial infection with *P. gingivalis* at 24 weeks. d) Lanes corresponding to Brain 1, 2, 5, 8, 9, 11 demonstrated a band at 400 bp. $p = 0.006$. e) Lanes corresponding to Brain 1, 2, 3, 4, 5, 6, 8, 9, 10, 11 demonstrated a band at 400 bp. $p = 0.0001$. f) Lanes corresponding to Brain 8 and 10 demonstrated a band at 400 bp.

The relationship between amyloid-beta and P. gingivalis

Microglia are the brains resident macrophages that have a central role protecting the brain from bacterial invasion [34]. For this guardian role, microglia possess danger associated molecular patterns (DAMPs) or pathogen recognition receptors (PRRs) [34–36]. There is support for the concept of *P. gingivalis* acting as a risk factor for cognitive deficit through manifestation of humoral immune responses [37, 38], also confirmed by Velsko et al. [27] in the ApoE$^{-/-}$ experimental periodontitis/atherosclerosis mouse model in which, not only the titre of antibodies to *P. gingivalis* were raised but atherosclerosis also formed [27, 30, 31] implying the pathological changes in blood vessel walls will have inevitably contributed to compromised blood flow to the brain. Poole et al. [33] confirmed the oral infection mediated inflammasome was being assembled in the mouse brain in the form of complement activation, (also implies indirect cytokine liberation), resulting from *P. gingivalis* invasion. However, due to the complete lack of ApoE protein in this animal model, the full AD inflammasome assembly comprising of Aβ foci with associated glial activity, remains under investigation.

The implications for the direct entry of *P. gingivalis* has for neurodegeneration is two-fold. In the

Poole et al. [14, 33] investigations, LPS from the bacterial cell wall will have elicited a local inflammatory reaction causing microglia to become reactive by expressing *de novo* immune markers including MHC class II proteins (allowing antigen presentation), and cytotoxic molecules (cytokines, complement) to migrate towards the pathogen in an attempt to clear it from the brain. Since the ApoE$^{-/-}$ experimental periodontitis model [27, 33] cannot form Aβ foci, we are unable to provide the relevant link between Aβ and *P. gingivalis* to tie in with the full AD inflammasome assembly in AD [8, 33]. However further research is going on in our laboratories to elucidate the full relevance of Aβ related inflammasome formation with *P. gingivalis* and its virulence factors. Currently, the only documented effect from introduction of LPS from Gram negative bacteria *in vivo* is provided by Sheng et al. [39], who generated secondary inflammatory mediators that increased the breakdown of APP resulting in extracellular deposits of insoluble neurotoxic oligomeric forms and polymerised Aβ foci in the brains of transgenic AD animal model.

Historical associations of microbial infections with onset of dementia

If dentistry evolved from medicine, then there is little surprise in the vision of the early medical/dental scholars held in view of the prevalent bacterial infectious diseases at the turn of the century. In 1913, Noguchi and Moore [40] demonstrated the spirochete *Treponema pallidum* [*T. pallidum*] infection in the brains of patients who died of a condition described as general paresis. This condition progressed to mental deterioration of the infected individual that describes dementia with typical clinical symptoms of poor memory, disorientation, and confusion [40]. This firmly established the concept of a link between peripheral bacterial infection of the brain and dementia. Proving infection related aetiology for a given neurodegenerative condition is no mean scientific task! However, Hill's criteria of causation is a research tool used in epidemiological studies to establish scientifically valid commonalities between potential causal agents and disease and is fully described in a review by Miklossy [41]. A century later, using historical observations and satisfying Hill's nine criteria of causation, Miklossy [11], demonstrated chronic spirochetal infections of the brain reproduce the neuropathological hallmarks of AD. The major significance of this seminal report [11] is that it paves the way for the acceptance of peripheral bacterial infections of the brain albeit as atrophic form of general paresis, resulting in dementia.

The "focal infection theory" on the other hand, implies that the oral microbes and their virulence factors affecting teeth and gingivae mobilise from the mouth and are likely responsible for causing infections elsewhere in the body [42]. William Hunter [43], a strong supporter of the concept observed that the origins of caries, pulpal necrosis and periodontitis were all microbial and proposed that these microbes may affect the health of remote body organs in the form of systemic diseases. Several decades later, a study by Kondo et al. [44], identified a number of risk factors contributing to the development of dementia in the Japanese population, one of which is an individual having fewer teeth in later life. Although, tooth loss is not currently regarded as a disease *per se*, the consequence of having fewer teeth with profound effects on memory in the later parts of life forms an interesting concept. A subsequent study involving Australian patients suffering from dementia showed various forms of dental disease, including caries and periodontal disease, to co-exist in their dentition [45]. However, it was Stein et al. [46] who singled out periodontal disease (PD) with the missing 3rd molar, linking it to the development of cognitive deficit. The same authors have subsequently demonstrated the effect of the Apoε4 allele, which besides advancing age, is a major genetic risk factor for developing LOAD [47] also correlated with fewer teeth, and development of a decline in memory earlier than the control individuals with either one or no risk factors [37].

The concept of peripheral infection and/or inflammatory mediators accessing the brain became better accepted once the circumventricular areas of the brain were reported to be free of the blood-brain barrier (BBB) [48, 49]. This enabled demonstration between the associations of peripheral infectious episodes and associated inflammatory burden in the elderly to deteriorating memory and the increased likelihood of being diagnosed with dementia [50–52]. Kamer et al. [53] provided experimental evidence in support of the peripheral inflammation from gingival infections in AD. The theme of peripheral inflammation originating from oral bacteria continued with Noble et al. [37], who reported a correlation of impaired cognition in subjects who had high titres of circulating IgG. These aforementioned investigations were further strengthened by a report from Sparks Stein et al. [38]

suggesting high circulating IgG tires to *P. gingivalis* are a risk factor for the development of AD. The missing piece of the zig-saw puzzle in the link between periodontal bacterium *P. gingivalis* and LOAD was provided by Poole et al. [14] who examined periventricular brain samples donated by ten patients with neuropathologically confirmed AD and age-matched clinically and neuropathologically normal controls, obtained from the Newcastle Brain Tissue Resource. This study exclusively demonstrated the presence of lipopolysaccharide (LPS) from *P. gingivalis* in brains from subjects having suffered from AD and was statistically significant ($p = 0.029$) over age-matched control brains (Fig. 3) and that the LPS was opsonized to glial cell surface membranes (Fig. 4b) supporting their continued priming in the maintenance of inflammation. More recent reports relating infections to a causative role in the onset of dementia are supported by Kamer et al. [54] suggesting that mild periodontitis is associated with higher brain amyloid load in normal elderly subjects in the hippocampus. It has been known for some time that the hippocampus is

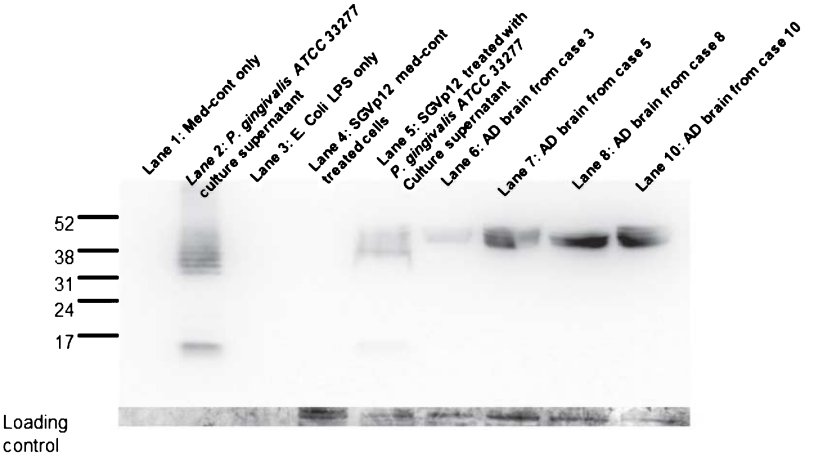

Fig. 3. Taken from Poole et al. [14]. Immunoblots to demonstrate LPS in human AD brain tissue. Total protein/lane (60 μg) was loaded on a 12.5% SDS-PAGE gel followed by a successful transfer to a PVDF membrane. Immunoblotting using the primary antibody (anti-*P. gingivalis* clone 1B5) and secondary detection using goat anti-mouse conjugated to HRP. Shows medium control (lane 1), failed to produce any bands whereas the positive control culture supernatants from *P. gingivalis* ATCC 33277 (lane 2) demonstrated a number of bands (45-12 kDa) corresponding to LPS in *P. gingivalis.* (b) *E. coli* LPS (lane 3), and cells treated with medium control (lane 4) showed no bands. The result in lane 5 confirmed the *de-novo* antigen detected by the aforementioned antibody was LPS on SVGp12 cells. Anti-*P. gingivalis* antibody (1B5) detected bands characteristic of the LPS at the expected molecular weight in AD case numbers 3, 5, 8 and 10. The loading control shows the protein was loaded in all test lanes.

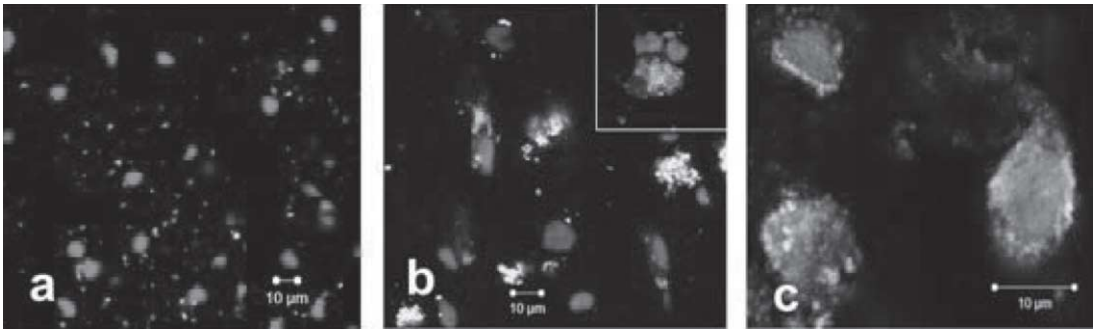

Fig. 4. Taken from Poole et al. [14]. Human AD brain. Confocal microscope images captured from snap-frozen brain tissue sections from Alzheimer's disease (AD) showing nuclei due to propidium iodide (PI) uptake. The images are overlaid with PI and the FITC signals. a) Negative control, primary antibody omitted. b) Immunolabeled using the anti-*P. gingivalis* (clone 1B5) antibody overnight at 4°C followed by detection using goat anti mouse FITC. Insert shows extracellular aggregates with granular (pebbly) appearance embedded within a smoother matrix. c) An adjacent section from the same brain labeled with mouse anti-CD14 for surface membrane labelling.

vulnerable to damage and Montagne et al. [55] have recently identified a vulnerability linked to the leaky BBB in elderly human subjects around the CA1 and the dentate gyrus subfields. It is however, unclear as to the timing of, and the cause of this perturbation in the integrity of the hippocampal BBB. The importance of this finding is that the very centre of the brain, which is associated with learning and memory, also demonstrates a high burden of neuropathological hallmarks in AD [6].

Periodontal bacteria are implicated in the development of vascular pathology [56–60] and that vascular lesions also co-exist with cognitive decline in the elderly associated with cerebral subcortical small vessel disease [61] which also shows loss of BBB integrity [62]. This leads to the proposal that chronic gingival infections could play a role in weakening the BBB via endotoxin (proteases) release during life. Bacterial endotoxins, in the systemic system together with circulating inflammatory mediators and their seepage into the brain is likely to initiate intracerebral inflammatory responses that may further contribute to potential mechanisms of disease pathology such as bacteraemia, systemic inflammation, BBB erosion, initiation of intra-cerebral inflammatory responses and tissue injury, dyslipidaemia and proteostasis specific to the disease condition [62].

Periodontal disease

Periodontitis is a polymicrobial dysbiotic inflammatory disease of the tooth supporting structures in humans [18, 32]. It is characterised by the destruction of cementum, periodontal ligament, and gingival connective tissue attachment to the root surface and adjacent alveolar bone. Periodontal disease involves complex synergistic interaction of numerous subgingival bacteria *Agregatibacter actinomycetemcomitans* [*A. actinomycetemcomitans*], *P. gingivalis*, *Treponema denticola* [*T. denticola*], *Tannerella forsythyia* [*T. forsythia*], and *Fusobacterium nucleatum* [*F. nucleatum*] [25]. Recent reports investigated the effects of gingival infection in apolipoprotein E gene knockout ($ApoE^{-/-}$) mice with selected periodontal bacteria (*P. gingivalis, T. denticola, T. forsythia*, *F. nucleatum*) as mono- and polymicrobial infections [27, 30, 31, 63–69].

Overall Lalla et al. [30], Velsko et al. [27, 65, 66] and Chukkapalli et al. [66–68] demonstrated, successful establishment and progression of PD (characterised by alveolar bone resorption and intrabony defects) in the $ApoE^{-/-}$ mouse with chronic gingival infection of two periodontal pathogens (*P. gingivalis*, *T. denticola*, *T. forsythia* and *F. nucleatum*) compared to sham-infection. Elevated serum pro-inflammatory cytokines (IL-1α and IL-1β) and IgG responses to bacterial infections were recorded [27, 64–68]. The pro-inflammatory cytokines and the humoral responses generated in each of the monoinfected mice provided further evidence of manifestation of chronic inflammation.

As proof-of-concept in the murine $ApoE^{-/-}$ genotype gingival infection model of Velsko et al. [27], Poole et al. [33] employed sensitive polymerase chain reaction (PCR) and sequencing to demonstrate *P. gingivalis* mobilization from the gingival tissue to the brain (Fig. 2) [33], likely via the haematogenous route [27], although other routes (Fig. 4) are also plausible [70]. Furthermore, the same study demonstrated hippocampal CA neurons were opsonised with complement activation fragments iC3b/C3b/C3d [33] (Fig. 5). Recent *in vivo* [fluorescence *in situ* hybridization or FISH] studies demonstrated the active invasion or oral bacteria (*P. gingivalis*, *T. denticola*) in gingival epithelium, aortic adventitial layer [27, 64–68]. In addition, periodontal bacteria have the potential to enhance systemic inflammatory atherosclerosis risk markers including serum amyloid A, nitric oxide, oxidised low density lipoprotein (LDL), lipid peroxidation, and a significantly increased serum lipid profile (cholesterol, triglycerides, chylomicrons, very (V)LDL, LDL, high (H)DL) suggesting altered cholesterol metabolism and potential for aortic and neuroinflammation [27, 64–68]. Our data confirmed the ongoing innate immune system activation and that infection is a critical risk factor for developing AD inflammatory pathology in the $ApoE^{-/-}$ phenotype (Fig. 5). Although a strong link between oral health and cardiovascular disease has been proposed for more than a century, but it is only since 2012, that the American Heart Association (Scientific Statement) officially recognised an association between periodontal diseases and the atherosclerotic vascular disease (ASVD). This recognition is a milestone scientific achievement indicating the relationship between periodontal disease and ASVD is potentially of great public health importance because of its high prevalence world-wide [71]. Similarly, with compelling evidence emerging from periodontal disease, vascular lesions co-existing with cognitive decline in the elderly associated with Aβ plaques [61] and/or stokes will one day be accepted as co-morbid states by the Neuropathology Society.

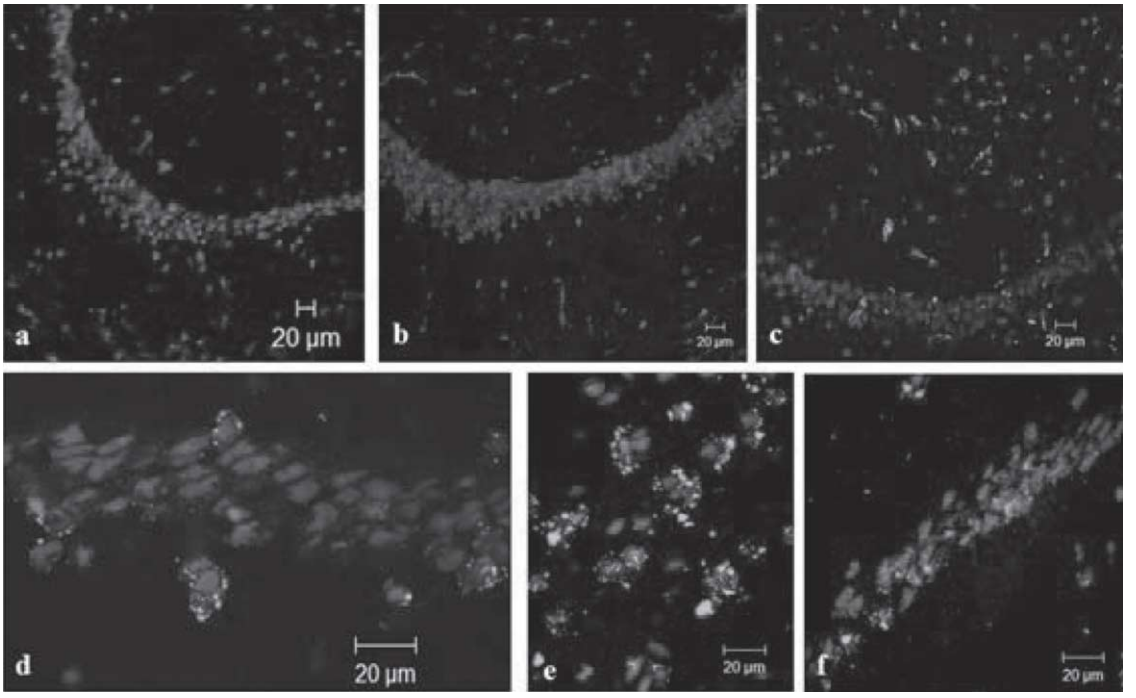

Fig. 5. Taken from Poole et al. [33]. Immunodetection of complement fragments in brain tissue sections using rat anti-mouse C3b/iC3b/C3d. (a) Negative control (b-c) sham-infected brains with rat anti-mouse C3b/iC3b/C3d (b) and rabbit anti-rat C9 neoepitope (c). (d–f) *P. gingivalis* infected brain with rat anti-mouse C3b/iC3b/C3d (d and e) and rabbit anti-rat C9 neoepitope (f); showing labeling on the cell surface membranes of the CA neurons in the infected brains ($p = 0.032$).

Anatomical relationship of facial nerves and the blood supply to the brain

The position of the oral cavity, serving the need for speech and food consumption, connects with the brain via series of nerves. Cranial nerve 1 (CN1) is the special sensory nerve for olfaction and contributes not only to our sense of smell but also to that of taste. Cranial nerve 1 has complex pathways that trigger visceral responses (salivation and nausea or accelerated peristalsis in the intestinal tract and increased gastric secretion) to various odors. Although CN1 is recognized and named as the olfactory nerve, the majority of the olfactory tract comprises of secondary, rather than primary sensory axons; thus it is really not a "nerve" but rather a bulb and tract. There is a physical connection between the oral and nasal cavity, extending onto the superior nasal conchae and nasal septum and contains neurosensory cells and olfactory glands, which keep the mucosa moist and in which the dissolution of inhaled scents (aromatic molecules) occurs. The peripheral processes of the primary sensory neurons in the epithelium perform as sensory receptors and transmit sensation centrally, which congregate into around 20 bundles, which, in turn, pass through foramina of the cribriform plate of the ethmoid bone. The cribriform plate of the ethmoid bone is the porous barrier between the nasal passages and the brain itself. Once they have passed through the cribriform plate, the central processes synapse on the secondary sensory neurons in the olfactory bulb itself, which houses the nerve cell bodies. Behind this area is the olfactory tract and trigone; the nerve cell bodies travel to the three olfactory areas, located in the anterior part of the entorhinal cortex area, encompassing the hippocampal gyrus and all ultimately lead to the hippocampus [72].

Cranial nerve V (CN V) or the trigeminal nerve, arising from the mid-lateral surface for the pons, is primarily a general sensory nerve with smaller motor component. There are three divisions of the CN V which are ophthalmic (V I), maxillary (V_2) and mandibular (V_3) where, the motor root of CN V travels with the mandibular branch. The ophthalmic division (V I) exits the neurocranium through the supraorbital fissure, the maxillary division (V_2) through the foramen rotundum in the sphenoid bone and the mandibular (V_3) branch through the sphe-

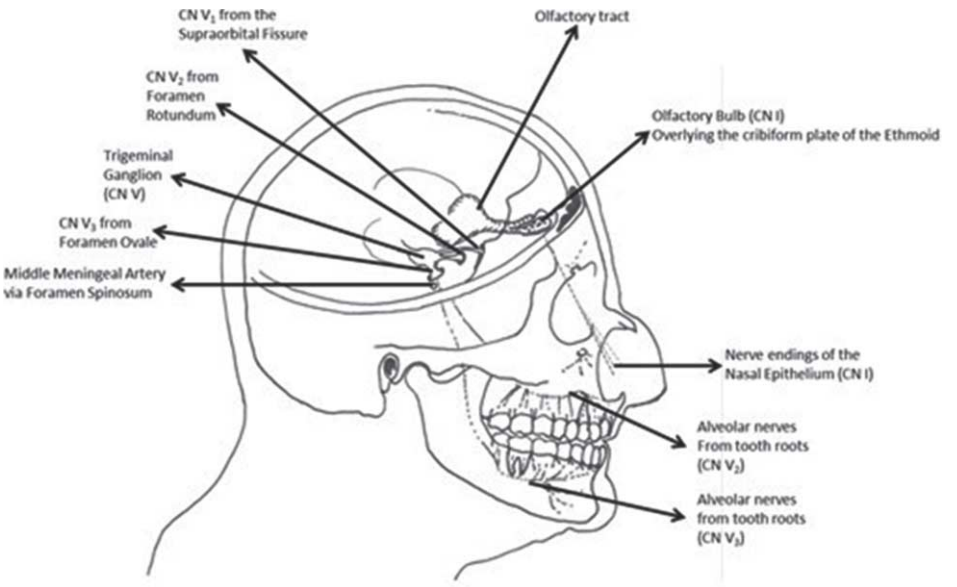

Fig. 6. From Singhrao et al. [71]. Nerve pathways from the oral and nasal cavity to the brain, showing the 2nd and 3rd branches of the Trigeminal (CN V) and the Olfactory nerve (CN I). The middle meningeal artery enters the brain at the foramen spinosum in the lateral portion of the greater wing of the sphenoid, and then follows the cranial base and lateral potions of the vault, supplying the dura and bones of the calvarium.

noid's foramen ovale. Cranial nerve V is a general sensory nerve to the scalp, face, nasal and oral cavities (including the teeth and tongue), and brachial motor nerve to the muscles of mastication (temporalis, masseter, medial pterygoid, and lateral pterygoid), tensor tympani, tensor (veli) palitini, mylohyoid, and the anterior belly of the digastric (Fig. 6). When dental or periodontal therapy is performed using local anesthesia (e.g., novocaine, xylocaine), the drug is injected into the oral mucosa covering the bony foramina where the sensory branches of the CN V exit into the oral cavity. For the maxillary dental arcade, the injection is aimed toward the pterygopalatine ganglion; for the mandibular teeth, this is directed toward the mandibular foramen. In the case of the pterygopalatine ganglion, this supplies sensation via branches of V_2 from the nasal cavity, plate, nasopharynx, and maxillary teeth. The lingual and inferior alveolar nerve branches carry sensation from the entirety of the lower jaw, mandibular teeth, gums, and anterior two thirds of the tongue as shown in Fig. 6. As with most nerves, the branches of the trigeminal nerve are accompanied by veins and arteries along the peripheries of their pathways [72] (Fig. 6).

The olfactory and the trigeminal nerve(s) pathways are also exploited by periodontal pathogens as a means of bypassing the BBB for direct entry into the CNS [13, 73, 74], an observation supported by studies in immunosuppressed animal models using *T. denticola* [75]. The animal model study allows some insight into the virulence of the organism and the host's immune defenses as being important for this occurrence.

The intravascular dissemination as an alternative mode of bacterial entry into the brain is favored due to bacteremia as mentioned earlier, association of periodontal pathogens with atherosclerotic lesions and in particular *P. gingivalis* having the ability to adhere to erythrocytes for innate immune evasion [57, 76, 77] as well as gaining advantage for transportation to remote body organs [77].

The brain is supplied by three paired blood vessels: the right and left internal carotid arteries, arising from the common carotid artery at the base of the neck. It has three divisions that enter the cranium, anteriorly through the carotid canal of the temporal bone and through foramen lacerum in the middle cranial fossa. The vertebral arteries arise from the subclavian arteries, bilaterally and both enter the cranium via the foramen magnum. The vertebral and internal carotid arteries unite on the base of the brain at the Circle of Willis, via a series of interconnecting smaller arteries. The basilar artery is created when the vertebral arteries join. The Circle of Willis itself is composed of the posterior cerebral, posterior communicating,

internal carotid, anterior cerebral and anterior communicating arteries; all these arteries branch to supply the brain itself [72] including the circumventricular organ regions where bacteria and bacterial products access the brain (Fig. 6).

Amyloid-beta as an immune molecule

An alternative hypothesis for the role of Aβ in subclinical and/or clinical AD individuals is that Aβ is acting as an antimicrobial peptide [78] to counteract infections by functioning as part of the early innate immune defense mechanisms that mediate innate and adaptive immune responses [79]. Traditionally, antimicrobial peptides act as look-outs for invading microorganisms to maintain the balance between commensals. The main target for antimicrobial peptides is the pathogen cell membrane, as most antimicrobial peptides are cationic [80]. Antimicrobial peptides undergo electrostatic interactions with negatively charged molecules to penetrate bacterial cell walls, including anionic lipids and LPS [80]. They then invade the lipid bilayer, creating transmembrane pores through which leakage of ions and metabolites, cytoplasmic components, dissipation of electrical potentials, and microbial lysis takes place [81]. This hypothesis suggests the involvement of a pathogenic precursor in the initiation of Aβ release before inflammation becomes detectable in the presence of amyloid plaques. We support this hypothesis and propose a suite of susceptibility traits and immunosuppressive (stressed or rundown) episodes during life that give way to chronic bacterial infections such as oral bacteria that cause periodontitis [18]. These bacterial elements in the individuals with susceptibility profiles may initially trigger damage to the BBB via release of their proteases and increase Aβ release to neutralize the effect of the pathogen. Over time Aβ will accumulate in the brains of healthy but susceptible individuals and initiate neuroinflammation that may cross the threshold from subclinical to LOAD.

Genetic risk factors for late-onset Alzheimer's disease and periodontitis

The APOE gene is a known genetic risk factor associated with LOAD, and more recent investigations suggest further genetic risk factor associations with innate immune molecules and inflammatory traits [4, 5, 82]. In particular, cytokine-related genes appear to be involved in the susceptibility to inflammation in LOAD [82–84] as well as in periodontal disease [85–87].

As the immune system plays a central role in periodontal disease pathogenesis [88], it is thought that periodontitis itself may have genetic associations. Polymorphisms in interleukin (IL)-α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α genotype are reported for periodontitis [85–87], and similarly IL-1α, IL-1β, IL-6, TNF-α, α2-macroglobulin, and α1-antichymotrypsin are all upregulated in AD [83, 84] suggesting commonalities between susceptibility profiles in these two disease conditions. As mentioned earlier, offspring of parents with AD have higher inflammatory cytokines in their blood than those who are descendants of non-AD parents [82]. Similarly, parents with poor oral health tend to have children with poor oral health; however, it is difficult to conclude that the poor oral health trait is a result of the genetic makeup of the individual and not simply an environmental influence [89].

Age-related personal hygiene changes as risk for infections

Advancing age is the greatest risk factor for all forms of AD. Some consequences of advancing age are a compromised immune system [90, 91] and a neglect of general and oral personal hygiene [46, 92, 93], and such conditions are associated with recurrent, chronic infections. Recurrent, chronic infections enhance systemic hyperinflammatory profile that may lead to confusion and other dementia-like clinical features [50–52] in which the exact structural/cellular changes taking place at the time remain unknown.

Several studies support deterioration in oral health with increasing age [94–97]. The exact reasons are poorly understood, but advancing age is likely to compromise the manual dexterity of senior citizens and this may make cleaning their teeth more difficult, or perhaps it is because as general health concerns and conditions increase with age, maintenance of oral health becomes a lower priority. The elderly are more likely to be on multiple medications, many of which, as a side effect, cause xerostomia and this will inevitably be a factor in deteriorating oral health [98]. Furthermore, if the elderly suffer from physical impairments, accessing the dentist may become more difficult. Elderly people resident in care institutions are, to a certain extent, dependent on the level of care within the establishment for the level of oral hygiene and dental health they receive. These factors

were supported in a large-scale survey carried out in the US by Griffin et al. [96], which found that older age groups were more likely to be edentulous or have untreated dental disease and root caries. Those who were either residents in institutions or homebound had higher levels of untreated cavities, gingivitis (a marker of poor oral hygiene), and poorer overall oral health than the elderly living independently. The study shows that cost, lack of transportation, and limited mobility were key barriers to accessing dental care for nursing home residents [96]. Other groups of elderly that show higher untreated dental disease and lower levels of oral health are those from ethnic minorities and low-income families [96].

The association between periodontal disease and Alzheimer's disease

It has been hypothesized that an oral infection may be a risk factor for the development of AD [99] and supporting longitudinal studies have shown that people with periodontal disease who progressed to AD had poorer oral health [46, 93, 96, 100–102]. Does poor oral health always mean that the bacteria will disseminate to the brain even in AD patients? From controlled experiments using animal models, and that of Foschi et al. [75], indicate that the presence and motility of the low virulence strains of periodontal bacteria may not be sufficient for them to access the brain. However, animal models of oral diseases (periodontitis and endodontic) may require an adjustment for the optimization of dosage and/or duration of infection to allow for detectable numbers of bacteria to access the brain. Our data demonstrates that fimbriated *P. gingivalis* strain (FDC 381) accessed the brain of ApoE$^{-/-}$ mice following 24-week chronic gingival infection [33] while *P. gingivalis* (ATCC 33277) did not, even in SCID mice [75]. It therefore, appears that the greater virulence of fimbriated *P. gingivalis* (FDC 381) is a likely bacterial strategy that accounts for its adherence to erythrocytes for innate immune evasion, a process that has gained the bacterium an advantage for hematogenous dissemination [77], to the brain [33]. However, it should be noted that patients suffering from AD are immunocompromised and demonstrate cognitive impairment implying poor management of oral health is to be expected and that together they can enhance the subgingival infection load and exacerbate periodontitis.

Nutritional deficiencies are documented in the elderly as well as in the dementia subjects, especially with regard to lessened intake of B-vitamins and folic acid in the diet. The marker that indicates these deficiencies also correlates with cognitive decline, but as consequence of disease rather than a cause [103]. The mechanism of cognitive decline is suggested via synaptic dysfunction, which is one of the earliest structural defects associated with decline in memory [104]. Diet provides the essential B-vitamins, phospholipids, and other micronutrients, which are required for the formation of new synapses [105].

Epidemiological evidence for the association between periodontitis and Alzheimer's disease

Several clinical/epidemiological studies have reported the relationship between poor oral health, edentulism, and poor memory [37, 46, 106, 107]. Further studies have examined possible inflammatory biomarkers in an attempt to link and/or to find new diagnostic makers of AD. Others have, however, used more specific measures including IgG levels to *P. gingivalis* and other specific periodontal bacteria [38, 53]. A study by Sparks Stein et al. [38] used cohort methodology analyzing levels of serum antibodies to periodontal disease. At the start of the study period, all participants were cognitively intact, but higher levels of serum antibodies to periodontal bacteria at baseline led to some individuals developing AD [38]. As baseline measures were taken years before diagnosis of AD, the elevation in serum antibodies cannot be attributed to secondary effects of AD (for example, poor oral hygiene). Although clinical measurements of oral health were not taken in the Sparks Stein et al. [38] investigation, periodontal bacterial species are generally accepted as being specific enough to periodontal disease and assessing serum antibody levels to these pathogens may prove to be a true indicator of periodontitis in AD patients.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this chapter.

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Section 5
Viral infections and Alzheimer’s disease

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Herpes and Alzheimer's Disease: Subversion in the Central Nervous System and How It Might Be Halted

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Abstract. The last 8 or so years have seen a large increase in the number of studies supporting the concept of a major role for herpes simplex virus type 1 (HSV1) in Alzheimer's disease (AD). The main advances have been made through studies in humans and in mice, investigating the likelihood of reactivation of the latent virus in brain. Others have aimed to explain the mechanisms in cells whereby the increase in amyloid-beta (Aβ) production on HSV1 infection of cells and mouse brains occurs, and the reason that infected cells make this increase. The possibility that other herpesviruses are involved in the development of AD has been explored, and human herpesvirus type 6, Epstein-Barr virus, and cytomegalovirus, in particular, have been implicated. Epidemiological studies have further supported the role specifically of HSV1 and its reactivation in the disease. Antiviral studies have continued, comparing those acting by different mechanisms, such as restricting viral replication, or blocking viral entry into cells, to treat HSV1-infected cell cultures, and then examining the extent to which the virus-induced increases in Aβ and AD-like tau are reduced. All the studies support the usage of antiviral treatment to slow or halt the progression of AD.

Keywords: Alzheimer's disease, amyloid-beta, antivirals, brain, epidemiology, herpes simplex virus type 1, virus reactivation

In the last eight years, there have been many major findings in research on the possible links between the common virus, herpes simplex virus type 1 (HSV1), and Alzheimer's disease (AD), and hence a large increase in the number of publications, which now total about one hundred, while relevant reviews number at least twenty. All these new articles support either directly or indirectly the concept of a major role for HSV1 in AD, and are especially convincing as they are based on widely differing types of experimental approach. Those published up till 2014 have been described in detail in a recent review [1], so this article will concentrate mainly on articles published subsequently.

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The concept of a viral role in AD states that in HSV1-infected people (who comprise 80–90% of the population by the age of 60, in most countries), the decline of the immune system with age enables HSV1 to travel from the peripheral nervous system (PNS) to the CNS (or possibly instead, it enters the brain as a new infection via the olfactory route). HSV1 then remains in brain in a latent state, but can be reactivated, as in the periphery, by events such as immunosuppression and stress. During each reactivation, the virus infection becomes productive, though presumably very localized–in effect a type of "mild" encephalitis (see below), with consequent neuronal damage. Recurrent reactivation results in accumulation of damage, leading eventually to the development of AD in the brain of those who carry an APOE-ε4 allele, accounting for some 60% of AD sufferers [2]. Similarly in the PNS, HSV1 reactivation

from latency is known to cause cold sores (herpes labialis), but mainly in those carrying an apoE-ε4 allele [2] (a result later confirmed by Koelle et al. [3]), paralleling the HSV1-APOE-ε4 connection in the CNS.

REACTIVATION OF HSV1 IN BRAIN

One of the most important aspects of the concept is that of reactivation of HSV1 in brain. Several studies were described in detail in the recent review [1], including a finding that provides major direct evidence of reactivation in brain: an examination of CSF samples sent to a reference laboratory for HSV testing [4], revealed, unexpectedly, that 26 of the 3200 samples were positive for the viral DNA. HSV DNA is present in CSF of herpes simplex encephalitis (HSE) patients but the disease could not explain the relatively very high proportion of samples that were viral DNA-positive (8 per thousand) as the prevalence of HSE in the population is much lower, only ∼2 per million. A further reason why HSE could not account for the results is that after HSE, HSV1 DNA remains in the CSF only briefly, for about a week (in contrast to the long life of intrathecal antibodies to HSV). The data thus not only confirm HSV1 presence in brain but also suggest that HSV1 reactivation in brain is not so infrequent. The finding is consistent with an earlier study using *in situ* hybridization, which suggested that immunosuppression causes latent HSV to reactivate and that subsequent replication leads to its amplification [5]: HSV DNA was detectable in postmortem brain specimens of subjects who had been immunosuppressed and were seropositive for HSV, but not in those who were seronegative or who had not been immunosuppressed.

Another relevant early study [6] described cases of "mild" encephalitis: patients' symptoms were less severe than usual and recovery was almost complete, with only minor sequelae, even though the patients were not treated with antivirals. In the days before antiviral treatment became routine, most cases of HSE were usually fatal, and those who survived often suffered severe neurological problems, including memory loss. Klapper et al. referred also to recurrent HSE, i.e., reactivation of latent HSV1 present in brain, and they suggested that other recurrences might not always have been recognized. They presciently speculated: "Is it possible that one or more reactivation events [of HSV1] resulting in mild disease could play an etiological role in such conditions [chronic psychiatric illness]"? Cases of recurrent HSE are still quite often reported, but of course they would not be seen by neuropathologists, who examine only fatal cases, i.e., the most severe, and who might well conclude therefore that "mild" encephalitis does not exist.

In mice, HSV1 latency in brain is established a few weeks after inoculation of the virus. Many early investigations indicated that its reactivation in brain was far rarer than in the trigeminal ganglia (TG), as determined by assays of dissociated and minced tissue such as the *ex vivo* (explant) assay of reactivation frequency (in which latently infected cells are cultured with susceptible uninfected ones). However, two recent very interesting studies indicate that HSV1 in mouse brain can in fact be reactivated relatively easily. Yao et al. [7] examined the brain stem and TG of HSV1-infected animals during latency and unexpectedly found in brain a greater number of copies of the viral genome, and also more frequent reactivation, than in the TG. They attributed this difference from previous results to a more rapid loss of viability of brain stem cells than of TG cells after dissociation, and especially after mincing, so that *ex vivo* measurements would not accurately assess viral reactivation in brain. The data of Ramakrishna et al. [8] were equally striking: they investigated HSV1-infected immunodeficient mice (lacking B and T cells) which were treated with intravenous immunoglobulin (IVIG) to promote long-term survival (via IVIG's immunomodulatory and antiviral activities). After high dose HSV1 inoculation, mice in which viral latency had been established in brain showed spontaneous reactivation of the virus; this was suppressed by T cells but not B cells. Hyperthermic stress caused HSV1 reactivation in brain of most of the animals, with subsequent occurrence of HSE.

Productive HSV1 infection causes damage via inflammatory processes as well as by direct viral action. Some of these processes can occur also during latency [9]: in HSV1-infected mice, several inflammatory markers such as toll-like receptor-4, interferon α/β, and p-IRF3, characteristic of viral replication, were all detectable in brain at a time well after virus inoculation, and therefore after the establishment of viral latency in brain, thus indicating that reactivation had occurred. The authors concluded that HSV-1 presence in the CNS could cause chronic neuroinflammation through recurrent reactivation, leading to activation of toll-like receptors and thence to cumulative neuronal dysfunction. All these data support the proposal that HSV1

reactivates – not just in the periphery, but also in the brain.

CELL BIOLOGICAL STUDIES

In another type of approach – cell biological studies aiming to find if the characteristic abnormal molecules found in AD brains can be produced by HSV1 infection – HSV1-infected cell cultures revealed accumulation of amyloid-beta (Aβ) [10–12], and of AD-like tau (P-tau) occurs [13–16]. Implicating HSV1 further in AD was the discovery that in AD brains, most of the HSV1 DNA is very specifically localized in amyloid plaques [17]: in brain of elderly controls, a much lower proportion of the viral DNA is present in plaques, presumably reflecting a lower extent of synthesis of Aβ, or else a more efficient removal of the peptide. The HSV1-induced increases in Aβ and P-tau were accounted for by increases in the relevant enzymes via, in the case of BACE, HSV1-induced PKR activation followed by phosphorylation of eukaryotic translation initiation factor 2-alpha (eIF-2α) [18]; eIF2α shuts off general protein synthesis, but reverses the inhibitory effect of the BACE1 5' untranslated region (5'UTR) in the BACE promoter on BACE expression. The PKR polymorphisms in AD patients discovered by Bullido et al. [19] could affect this process, thereby leading to the observed high level of activated PKR in AD brains.

Santana et al. [20] investigated the effects of mild oxidative stress combined with HSV1 infection of cells (which itself causes oxidation). They found that oxidative stress significantly augmented the HSV1-induced accumulation of Aβ and its secretion, as well as the inhibition of autophagy, although it did not increase the degradation of long-lived proteins. These oxidative effects were not attributable to enhanced virus replication as, surprisingly, oxidation reduced viral DNA replication and reduced even more the formation that leads to the neurodegeneration seen in AD.

Civitelli et al. [21] found that HSV-1 infection of cultured mouse cortical neurons and SH-SY5Y neuroblastoma cells causes the production of several APP fragments, including the APP intracellular domain (AICD). AICD binds the promoter region of both neprilysin (NEP), the major Aβ-degrading enzyme, and GSK3β, the enzyme causing hyperphosphorylation of tau. NEP level and enzyme activity were initially stimulated by infection but later were down-regulated. GSK3β level and activity remained almost constant, although at late stages of infection the enzyme was inactivated through being phosphorylated at Ser9. However, a second study by the same group [22] showed that HSV1 caused activation of phosphorylated GSK3. The activation of pGSK3 was Ca2+-dependent and was essential for the HSV-1-dependent phosphorylation of APP at Thr668, leading then to its subsequent degradation and to the intraneuronal accumulation of Aβ. A very significant finding was that HSV-1 infection reduced the expression of the presynaptic proteins synapsin-1 and synaptophysin, and depressed synaptic transmission. By using 4G8 antibody which binds to Aβ, and also by infecting APP-knockout mice, the authors showed that these inhibitory effects on synaptic function were dependent on GSK-3 activation and intraneuronal accumulation of Aβ.

The increase in Aβ that HSV1 causes raised the possibility that at least initially, the peptide at low levels might function as part of the innate immune system, acting protectively as a "bioflocculant", i.e., binding neurotoxic agents, as previously suggested by Robinson and Bishop [23], or as an anti-microbial peptide [10]; however, in the latter study, although Aβ appeared to have antiviral activity, it was attributable to its toxic effect on the cells. Furthermore, virucidal assays, which assess the capacity of the test molecule to inactivate virus particles, showed no effect on viral infectivity. However, in view of recent positive findings (see below), the antiviral activity is probably determined by the method of its preparation and its state of aggregation. In any case, though, Aβ eventually becomes toxic when over-produced and when oligomerization occurs.

DOES Aβ HAVE ANTIMICROBIAL PROPERTIES?

There is now evidence that Aβ, which structurally resembles antimicrobial peptides (AMPs) and, like them, can cause activation of immune cells, does indeed have antiviral activity. A number of studies have implicated certain bacteria – spirochetes [24] and *Chlamydia pneumoniae (C. pneumoniae)* [25], as well as HSV1, in the development of AD. Both types of bacteria elicit the formation of Aβ and P-tau, and components of both colocalize with AD pathology. The antibacterial activity of Aβ was detected first by Soscia et al. [26] and is discussed later in this section together with a recent study from the same group.

The first paper on the antiviral properties of Aβ [27] investigated its effect on influenza virus A during infection of several human and canine epithelial cell cultures, used as model systems. The authors found that the activity of $A\beta_{42}$ was much greater than that of $A\beta_{40}$, and that the maximum antiviral effect of $A\beta_{42}$ was achieved when it was pre-incubated with the virus, thereby indicating that it acts on the virus rather than on the cell. Also, Aβ caused aggregation of the virus, reduced viral protein synthesis, and modulated its interaction with phagocytes.

As to the effect of Aβ on HSV1, Bourgade et al. [28] infected cell cultures with the virus and found that both $A\beta_{40}$ and $A\beta_{42}$ inhibited HSV1 DNA replication when added to the cultures. This occurred either when the peptides were added before the virus or when added together with it, but not when added after virus addition. Also, in a cell-free system, Aβ interacted directly with HSV1 (as $A\beta_{42}$ did with influenza virus), indicating that in the cell cultures, it prevented HSV1 entry into cells. Both this and the influenza virus study showed also that Aβ acts selectively against enveloped viruses as opposed to non-enveloped viruses, and Bourgade et al suggested that this might reflect Aβ insertion into the viral envelope. In a second study, Bourgade et al. [29] used co-cultures of neuroglioma (H4) and glioblastoma (U118-MG) cells as an *in vitro* model, and found that the H4 cells secreted $A\beta_{42}$ in response to HSV-1 challenge, and that U118-MG cells could rapidly internalize $A\beta_{42}$. Extraneous $A\beta_{42}$ induced strong production of cytokines in the cell lines, and a combination of $A\beta_{42}$ and HSV-1 induced the production of the pro-inflammatory cytokines TNFα and IL-1β, and IFNα in the cell lines. $A\beta_{42}$-conditioned medium from HSV-1-infected H4 cells, when added to cultures of H4 cells, conferred Aβ-dependent protection against HSV-1 replication when the cells were challenged with HSV-1. The authors proposed that in human brain, $A\beta_{42}$ acts as an AMP against neurotropic enveloped viruses such as HSV1; also, in agreement with the present author's suggestions, they considered that eventual overproduction of Aβ peptide might contribute to amyloid plaque formation.

Intriguingly, α-synuclein (Asyn), another AMP-like peptide, has very recently been shown by Beatman et al. [30] to have antiviral activity against certain enveloped RNA viruses. Infection of primary neurons with West Nile virus (WNV) or with Venezuelan equine encephalitis virus caused an increase in Asyn expression, and infection of Asyn knock-out mice resulted in a huge increase in number of infectious viruses, and much greater subsequent mortality, compared with wild-type and heterozygous litter mates. The authors suggested that WNV-induced Asyn inhibits viral replication, growth, and injury in the CNS and that the peptide has a novel and important functional role in the development of Parkinson's disease.

Both the influenza and the HSV1 studies tested Aβ efficacy as an antiviral by assaying virus level, using quantitative PCR on viral DNA extracted from the cell cultures. However, PCR has the disadvantage of measuring DNA not only from "live" but also from inactivated virus, thereby over-estimating the virus level. Also, in the HSV1 studies, the Aβ concentration used was high (20 μg/ml) probably very much greater than the levels in brain cells. It would therefore be well worth extending the studies using a much lower Aβ concentration and assaying virus levels by standard virological methods, such as the plaque assay (the method used by Beatman et al. [30]).

Further strong evidence for the protective role of Aβ, although unexpectedly in its oligomeric form, has been obtained in an interesting, very detailed study by Kumar et al. [31]. This followed work by the same group examining the effect of synthetic Aβ on the growth of eight pathogens, the yeast *Candida albicans* (C.albicans) and seven common types of bacteria, in culture, which indicated that Aβ has a protective role in innate immunity [26]. In the more recent study, the microbes investigated were the bacterium, *Salmonella typhimurium (S. typhimurium)*, and the yeast *C. albicans*. The targets were transfected human neuroglioma cells (H4) over-expressing Aβ, transgenic (Tg) nematodes, *Caenorhabditis elegans (C. elegans),* expressing Aβ in body wall muscle, and Tg mice overexpressing Aβ. The authors showed that Aβ protected the cultures of transfected cells and also the Tg nematodes, greatly increasing their survival when infected by *C. albicans*. Similarly, the Tg mice survived infection with *S. Typhimurium* for a far longer time period than did wild-type and APP knock-out mice. To examine the protective mechanism, the authors compared Aβ with an antimicrobial peptide (AMP), LL-37, which is known to protect against microbes by oligomerizing and binding to their surface, thereby preventing their attachment to the target cells, and then forming fibrils round them so that they are immobilized. On infecting the transfected H4 cells with *C. albicans*, the authors found that the transfected cells bound fewer yeasts than did non-transfected H4 cells, and

that the Aβ bound to the yeast cell walls, but only if it was in oligomeric form; then, like LL-37, the Aβ wrapped up the yeast. Similarly, on infection of the nematodes, the yeasts became entrapped and the clumps thus formed were stainable with thioflavin S, as are amyloid plaques in human brain. Further, in Tg mice – animals that normally develop amyloid plaques only at a later age – plaques were seen in young mice at just 2 days after infection with *S. Typhymurium*. The authors commented that the same features, oligomerization, fibrillization, and carbohydrate binding, are associated also with the pathophysiological effect of Aβ, and they suggest that dysregulation of the normal protective activity of Aβ leads to AD pathology.

Unfortunately, the authors did not investigate infection with HSV1 in either study, despite its being the pathogen most frequently implicated in AD, and implicated via diverse approaches. In fact their immobilized pathogen model strikingly resembles the pictures of HSV1 DNA embedded within amyloid plaques in AD brains, a finding published in 2009 [17], and the bioflocculent model proposed by Robinson and Bishop in 2002 [23]. Further, the bacteria and the yeast investigated in the studies described above have never been associated in any way with AD, yet the two types of bacteria that are strongly implicated in the disease (spirochetes and C. *pneumoniae*) both of which, significantly, are intracellular, were not investigated.

HSE AND Aβ

Interestingly, a link between HSE and Aβ was discovered by Bearer et al. [32], who investigated autopsy brain tissue from three HSE patients: a 9-day-old, a 8-year-old, and a 76-year-old (the latter showing no evidence of AD). They detected Aβ but no P-tau in brain of each subject. Aβ was not detected in cases of non-herpetic viral encephalitis. They concluded that HSV can induce the formation of Aβ deposits, and recommended future follow-up of HSE patients who survive to find if the plaques and HSV1 infection persist, i.e., if more Aβ is deposited.

ARE THERE OTHER HERPES VIRUSES IN THE ELDERLY BRAIN?

There have been very few studies on the possible involvement of other herpes viruses in AD. Most of these viruses, if detected at all in brain, were found in a relatively low proportion of AD patients and elderly controls, compared to HSV1, apart from human herpesvirus type 6 (HHV6) which, in the author's laboratory was detected in brain of 70% and 40% of AD patients and age-matched controls respectively [28]. It was suggested that as there was considerable overlap of HHV6 and HSV1 in brain, HHV6 might act together with HSV1 in the development of AD. Previously, the same laboratory found no varicella zoster virus (VZV) in brain [34], but detected HSV2 in 13% and 20%, respectively, of patients and controls, cytomegalovirus (CMV) in 36% and 34%, respectively [33], and in 93% of vascular dementia patients [35].

Carbone et al. [36] sought the presence of the DNA of CMV, Epstein Barr virus (EBV), and HHV6 in peripheral blood leucocytes (PBL) and in brain. No CMV was detected in any samples, but EBV was detected in 45% of PBL from AD patients, 31% from controls, and in 6% of AD brains. HHV6 was detected in 23% PBL from AD patients, 4% from controls, and in 17% of AD brains. In subjects followed for 5 years, the percentage positive for EBV and HHV6 increased in those who developed AD, as did serum IgG levels for CMV and HHV6. They considered that the non-detection of CMV DNA, in contrast to their anti-CMV antibody detection and to the data of Lin et al. [33], possibly reflected the inability of their technique to detect low levels of CMV DNA. They concluded that EBV, HHV6, and perhaps CMV might all be implicated in the progression to AD.

In a later study [37], the authors examined AD patients and elderly controls over a five-year period for cognitive performance and for clinical diagnosis of AD, investigating genetic factors regulating antiviral response, such as IFN-λ3. They found that the genes responsible were associated with increased risk of cognitive decline and AD, again implicating EBV and HHV6, and they proposed that impaired immunity against persistent viruses, such as herpesviruses, in genetically predisposed elderly people might cause recurrent virus reactivation from latency, hence activating brain microglia, and increasing Aβ production and accumulation. An earlier publication, by Carter [38] had discussed putative antiviral host responses, specifically to HSV1, which could affect its infectivity or replication; these included nitric oxide, cysteine protease inhibitor cystatin C and certain cytokines, namely, IL1A, IL2, IL1RN, IL6, IL18, and TNF and as Carter commented, their effects would be influenced by any polymorphisms.

Recently, the effects of HSV2, another herpes virus highly homologous to HSV1, have been studied in

cultured human neuroblastoma cells [39]. HSV2 was found, like HSV1, to cause increased accumulation of abnormally phosphorylated tau and Aβ, altered APP processing, and impaired autophagy. The authors suggested that HSV2 (and other herpesviruses) might play a role in AD as it remains latent in sensory neurons but is capable of reactivating, and it can infect the brain and cause neurological symptoms, just as HSV1 infection does. However, they acknowledged that HSV2 usually causes HSE only in neonates, not in adults, and that serological data show that the virus infects a much lower proportion of the population, and resides in far fewer elderly human brains than does HSV1.

All these results, together with the discovery that in AD brains, almost all the HSV1 DNA resides within amyloid plaques [17], suggests that in many AD patients, HSV1 in brain is responsible for the abnormal processing of amyloid precursor protein (APP), for the formation of Aβ, its toxic aggregates, and of plaques, for abnormal phosphorylation of tau, and for synaptic dysfunction: the major features of AD. Whether the other herpesviruses contribute remains to be confirmed; possibly in the case of CMV, its action might be through immune dysregulation, as proposed by Stowe et al. [40].

EPIDEMIOLOGICAL STUDIES

There have been further epidemiological investigations on anti-HSV1 IgG and IgM antibodies in serum from AD patients. The rationale for the serum antibody work is that while the presence of IgG shows that the person has been infected with HSV1, the presence of IgM indicates that recent reactivation of HSV1 has occurred. However, serum antibody levels reflect the response to the virus in the periphery; whether or not they reflect response to the virus in brain is unknown because at present, no imaging method can detect either latent HSV1 in brain, or reactivated virus if present at very low levels. It does seem likely though that events that cause reactivation in the periphery, such as stress and immunosuppression, would cause reactivation also in the brain, but perhaps less severely.

Many antibody studies have shown an association between systemic infections and cognitive decline, with HSV1 as the main suspect [41–46] – but see comment on [46] by Itzhaki and Klapper [47]. Letenneur et al. [44], Feart et al. [45], and Lövheim et al. [46] mainly implicated IgM, thereby suggesting that HSV1 reactivations were the events leading to the development of AD, However, a second study by Lövheim et al. [49] found, surprisingly, an association of IgG, but not IgM, with AD, thus implicating HSV1 presence rather than activity in AD. This result contradicted the authors' previous data and those of others, so to explain the difference they suggested that it might result from the different approaches used – the previous ones being cohort studies, and their present one a case-control study. Alternatively, it could be because of HSV1 affecting an early stage in AD development, or perhaps it reflected their paucity of IgM-positive subjects.

Two investigations have been made on the possible association, in *young* subjects, of infection by a specific virus, or of infectious burden (I.B. – seropositivity to several microbes), with cognition or AD. One study investigated 612 soldiers in the Israeli military (59% male and 41% female, aged 19–21) for HSV-1 infection and possible association with cognitive functioning and language abilities [50]. After controlling for education, immigration status, and sex (although not for socio-economic status), and removing those with mild to moderate mental illness, the 62% who were seropositive for HSV-1 infection were found to have lower IQ and lower language skills. The second study on young to middle aged subjects [51] examined serum IgG antibodies to toxocariasis, toxoplasmosis, hepatitis A, hepatitis B, and hepatitis C, CMV, HSV1, and HSV2, in over 5,000 subjects aged 20–59 years. Cognition was assessed by three tests: the Third National Health and Nutrition Examination Survey computer-based simple reaction time (SRT), symbol-digit substitution (SDS), and serial-digit learning (SDL) tasks. The infectious burden index was found to be associated with two of the three cognitive function measures, SDS and SDL, on controlling for age, sex, race-ethnicity, educational attainment, and the poverty-to-income ratio (an estimate of socioeconomic status). HSV1, CMV, and hepatitis A were the main contributors to the association, that of hepatitis C was very low, and those of HSV2, toxoplasmosis, toxocariasis, and hepatitis B were intermediate.

Possible microbial associations with cognition or with AD in older subjects have recently been investigated in three studies. D'Aiuto et al. [52] used functional MRI to evaluate brain activation during a working memory task, and found an association between "nonencephalitic HSV-1 infection", assessed by serum IgG, and functional brain changes linked with working memory impairment. Barnes

et al. [53], in a longitudinal study, implicated CMV in an increased risk of AD, and stated that HSV1 was not related to AD incidence. However, Itzhaki and Klapper [54] pointed out that Barnes et al used a far less sensitive assay for HSV1 than for CMV, detecting only a single viral glycoprotein for HSV1 whereas for CMV, all of its proteins were detectable. From data obtained in another longitudinal study, Nimgaonkar et al. [55] maintained that CMV, HSV2, or *Toxoplasma gondii* exposure, but not HSV1 exposure, were associated with cognitive decline in older persons; however, in their discussion they alluded to the lack of sensitivity of their assays for HSV1, in any case adding that not finding an association between exposure to HSV-1 and cognitive decline did not preclude a role for HSV1. Such differences in sensitivity of assays used for detecting antibodies to various viruses should obviously be taken into account when comparing different viruses or estimating infectious burden.

FURTHER ANTIVIRAL STUDIES

Further investigations have been pursued on antiviral treatment of cells in culture during HSV1 infection, following the studies on acyclovir (ACV), penciclovir (PCV), foscarnet [56], and BAY 57–1293 [57], all of which inhibit viral DNA replication. Each of these agents greatly reduced HSV1-induced formation of P-tau and Aβ (and of HSV1, as expected), P-tau dropping to almost zero, but Aβ decreasing to 20–30% of the value without the antiviral. This showed that HSV1 DNA replication is needed for the abnormal phosphorylation of tau, but not for Aβ formation, so the decrease in the latter caused by the antivirals was attributed instead to the antiviral causing a reduction in viral spread, because of reduced viral replication. Another agent, IVIG, also reduced P-tau and Aβ, probably through preventing HSV1 entry into cells, and treatment with a combination of IVIG and ACV was found to be particularly effective [58]. The authors then tried a type of anti-HSV1 antiviral known to prevent HSV1 entry, namely, fucans-sulphated polysaccharides, which are derived from various types of brown algae. The most efficient of these in reducing P-tau and Aβ was an extract from *Undaria pinnatifida*, and this, when used in combination with ACV (even at a very low ACV dose, only one tenth of that in the ACV-PCV-foscarnet study), lead to a marked synergistic effect [59]. Fucans are much more readily obtainable than is IVIG, so that treating AD patients with the fucan from *Undaria* together with valacyclovir (the biodrug of ACV, which is far better absorbed in the body than is ACV) would be particularly suitable, as well as relatively inexpensive.

CONCLUSIONS

It is sometimes asserted that HSV1 presence in AD brain – the basis of the viral concept – and the effects of the virus, are a consequence either of the disease itself or of APOE-ε4 carriage conferring particular susceptibility to HSV1 infection of the brain. However, the former suggestion is rebutted by the fact that the virus is present in brain of a high proportion of elderly *controls* as well as AD patients, and the latter by the fact that many elderly controls harbor HSV1 in brain but only a few carry an APOE-ε4 allele [2]. Thus, the data strongly indicate that HSV1 is a cause, not an effect, of the disease (nor an effect of having the "wrong" APOE allele). Also, as mentioned above, the APOE-ε4-HSV1 association in cold sores (as well as APOE's influence on microbial diseases [60]) support the concept, as do the data described above and in previous reviews [1, 61], in particular, work on HSV1-APOE interactions [62–64]. And the diversity of the types of study lends further credence to the concept. Whether or not HSV1 acts in combination with another microbe is unknown but should be investigated. And whether HSV1 augments the effect of a non-microbial factor is unknown also, but cannot usefully be discussed, as no other factor has been proposed that is known to be more damaging specifically in those who will develop AD than in those fortunate enough to evade it. Whatever the answers to these questions, a clinical trial treating patients with an antiviral to slow or halt disease progression is now surely warranted.

DISCLOSURE STATEMENT

The author's disclosure is available online (http://j-alz.com/manuscript-disclosures/16-0607r1).

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Herpes Simplex Virus Type 1 Neuronal Infection Elicits Cellular and Molecular Mechanisms of Neuroinflammation and Neurodegeneration in *in vitro* and *in vivo* Mice Models

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Abstract. Herpes simplex virus type 1 (HSV-1) is a neurotropic virus able to establish a persistent latent infection in the host. Herpes simplex encephalitis (HSE) is associated with a high mortality rate and significant neurological, neuropsychological, and neurobehavioral sequelae, which afflict patients for life. Currently, it is unclear whether asymptomatic recurrent reactivations of HSV-1 occur in the central nervous systems in infected people, and if these events could lead to a progressive deterioration of neuronal function. In this context, HSV-1 constitutes an important candidate to be included among the risk factors for the development of Alzheimer's disease. Our group have demonstrated that HSV-1 triggers neurodegenerative events in *in vitro* and *in vivo* induced neuronal infection, evidenced by increase in tau hyperphosphorylation and caspase-3 dependent cleavage of tau protein, resembling what occurs in neurodegenerative diseases. In addition, in an *in vivo* model, a reactivation episode during asymptomatic latency of HSV-1 infection in mice was accompanied by upregulation of neuroinflammatory markers (toll-like receptor-4, interferon α/β, and p-IRF3). Besides, previous reports have shown that HSV-1 inhibits apoptosis during early infection, but is pro-apoptotic during productive infection. Taking in consideration that the stress sensors AMPK and Sirt1 are involved in neuronal survival and neuroprotection, we hypothesized that HSV-1 could activate the AMPK/Sirt1 axis as a strategy to establish latency through inhibition of apoptosis and restoration of the energy status. Thus, we demonstrated that HSV-1 modulates the AMPK/Sirt1 axis differentially during infection, interfering with pro-apoptotic signaling and regulating mitochondrial biogenesis, pivotal processes in the lifetime of neurons in the central nervous system. In conclusion, our findings support the idea that HSV-1 could contribute to induce neurodegenerative processes in age-associated pathologies such as Alzheimer's disease.

Keyword: Alzheimer's disease, AMPK/Sirt1, HHV-6, HSE, HSV-1, IRF3, latency, neurodegeneration, neuroinflammation, neurotropic virus, paired helical filaments, tangles, tau, TauC3, TLR2, TLR4

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a prevalent neurotropic virus that establishes persistent latent infection in sensory ganglion of infected individuals. Viral reactivation and recurrent clinical symptoms directly depend on the immunological state of the infected individuals. The pathogenic mechanisms of HSV-1 at the central nervous system (CNS) are not well known. However, accumulating data suggest that HSV-1 is able to establish latency in the CNS in humans [1–4], and that this condition would not be harmless. Indeed, it has been estimated that in approximately 70% of the population over 50 years old, the virus enters the brain and infects neurons [5], suggesting the existence of recurrent reactivations in infected individuals whose neuronal functions could be altered (for review see [6]). Nevertheless, the possibility that subclinical neurological deleterious effects caused by HSV-1 chronic reactivation events in the CNS could contribute to the development of neuropsychiatric disorders in elderly people has been underestimated. Currently, it is unclear whether a neuron that undergoes viral reactivation and produces infectious particles survives and resumes latency, loses functionality, or is killed [6–8].

The present review describes some aspects related to the cellular and molecular mechanisms of neuroinflammation and neurodegeneration triggered by HSV-1 neuronal infection. We comment and discuss the main contributions of our group on this interesting and scarcely studied research topic.

NEURONAL CYTOSKELETAL DYNAMIC MODIFICATION AND NEURODEGENERATION INDUCED BY INFECTION WITH HERPES SIMPLEX VIRUS TYPE 1

In neurodegenerative diseases, one of the earliest characteristic features detected is the loss of synapses and retrograde degeneration of neurons, which appears to be accompanied by a decrease of the intracellular transport and correlates with the incipient loss of memory and brain functions [9]. Several triggering events have been implicated in these effects, including oxidative stress, inflammatory cytokines, lack of growth factors, or the toxic amyloid-β peptide, which may lead to the decay of the axon or the neuron as a whole [9].

The aim of the Zambrano et al. [10] study was to determine whether disruption of microtubule dynamics and neurodegeneration processes occur in neuronal cells infected *in vitro* with HSV-1.

Microtubules play a central role in several neuronal functions such as the transport of recycled proteins, vesicles in the endocytic and lysosomal systems, and mitochondria from the axon terminal to the soma. Microtubules in neurite processes undergo multiple posttranslational modifications necessary to accomplish neuronal functions. For example, increased acetylated (Ac) tubulin is associated with microtubule stability and increased anterograde traffic flow [11–13], whereas tyrosinated (Tyr) tubulin is found in microtubules that are more dynamic.

Zambrano et al. [10] observed shortening of neuritic processes and reduction in neuronal viability induced by HSV-1 infection. The study demonstrated that HSV-1 (strain F) induced microtubule rearrangement in mice primary neuron cultures, which started at 4 hpi (hours post infection) (Fig. 1A). The first change observed was an increase in microtubular dynamics near the neuronal soma, suggesting cytoskeletal modifications, which are necessary for viral spread to the neuronal nucleus. Then, after 16 hpi, the changes occur in neuritic processes, increasing cytoskeleton stability (acetylated-tubulin) in surviving neurons, suggesting alterations in neuronal functions that would facilitate viral exit. Finally, after 18 hpi, 80% of neurons showed shortening in neurite processes and neuron viability was reduced to 40%. In agreement with these results, a previous study reported an increase of acetylated tubulin, at approximately 16 hpi either in COS (fibroblast-like cell line derived from monkey kidney tissue) cells transiently expressing VP22 (Viral protein 22) or in Vero cells infected with HSV-1 [14]. Besides, infection of Vero cells with HSV-1 induced microtubule reorganization, beginning at approximately 9 hpi and this correlated with the nuclear localization of the viral proteins VP22 [15], VP13/14, Vhs (virion host shutoff), and VP16 [16]. Thus, the active retention of these virion components by cytoskeletal structures may function to regulate its subcellular localization [15].

Taken together, these observations support the idea that HSV-1 plays a role in microtubule reorganization of neurons, keeping neuronal acetylated tubulin stabilized during the first hours of infection, with increased dynamics of microtubules (tyrosinated tubulin) near the soma, suggesting negative consequences in neuronal function in favor of HSV-1 replication. Then,

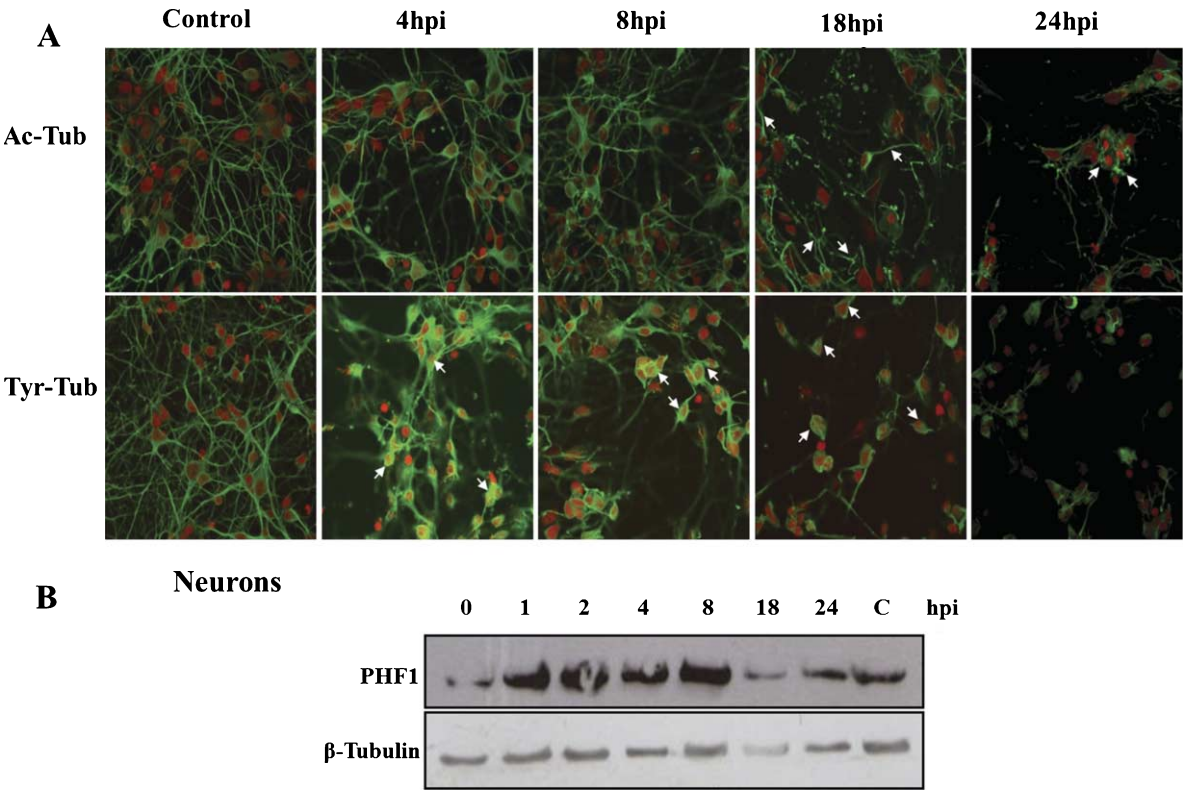

Fig. 1. A) Changes in neuronal cytoskeleton induced by HSV-1 infection. Primary cortical neurons, untreated controls and HSV-1-infected (moi 10) at 4, 8, 18, and 24 hpi, were stained with specific antibodies for HSV-1, acetylated tubulin (Ac-Tub), and tyrosined tubulin (Tyr-Tub). Nuclei were stained with propidium iodide. The results are representative of three separate experiments. Magnification 100X. B) Hyperphosphorylation of tau protein by HSV-1 infection. Western blot analyses show PHF1 and tubulin in primary cortical neurons untreated (C) and after 0, 1, 2, 4, 8, 18, and 24 hpi with HSV-1 (moi 10). Blots shown are representative of three separate experiments. Reprinted from [10], *Journal of Alzheimer's Disease*, Volume 14, Zambrano A, Solis L, Salvadores N, Cortes M, Lerchundi R, Otth C, Neuronal cytoskeletal dynamic modification and neurodegeneration induced by infection with herpes simplex virus type 1, pages 1–11, Copyright (2008), with permission from IOS Press.

after 16 hpi of viral infection, increased microtubule stability in surviving neurons may help viral exit from the cell, facilitating the transport of naked capsids to their point of envelopment within the cell [14]. In late HSV-1 infection (24 hpi), the global effects were disruption of neurite processes and neuronal death.

In order to further elucidate the neurodegenerative effect of HSV-1 on neuronal cells, Zambrano et al. [10] reported for the first time that HSV-1 infection induces hyperphosphorylation of tau protein. Hyperphosphorylated and aggregated tau (PHF-tau) are the major components of the paired helical filaments (PHFs) that make up the neurofibrillary tangles of neurodegenerative processes attributed to a number of neurodegenerative diseases such as Alzheimer's disease (AD), tauopathies, and Parkinson's disease [17–19]. The study demonstrated that HSV-1 triggered hyperphosphorylation of tau epitopes S^{202}/T^{205} and S^{396}/S^{404} in primary neuronal cultures (Fig. 1B). However, changes in tau hyperphosphorylation occurred during the first hours of infection, before neuronal death takes place. These results suggest a possible role for HSV-1 infection on neuronal cytoskeletal disruption and neurodegenerative processes, which *in vivo* could be magnified by recurrent viral reactivation episodes.

Repeated restraint stress in the rat has been shown to exacerbate neuronal damage in the hippocampus caused by a variety of excitotoxic and metabolic insults to the brain leading to memory loss, suggesting that stress can be a potent modulator of hippocampal degeneration [20, 21]. In this context, it has been hypothesized that hyperphosphorylation of tau may be an underlying point of pathological convergence for several neuropsychiatric disorders [22] or be involved in certain aspects of central processing of stress stimuli [20, 23]. Considering that (i) HSV- 1 infection is virtually universal and (ii) a declined

immune system in elderly people may allow the virus to reach the CNS, these results suggest that HSV-1 may progressively participate in neuronal damage in the brain of elderly people, contributing to neurological cognitive impairment.

In conclusion, these findings represent an important step in understanding how neuronal microtubular dynamics and neuronal integrity can be affected by viral infection, and they provide information that suggests a possible link between HSV-1 infection and neurodegenerative processes.

TAU CLEAVAGE AT D^{421} BY CASPASE-3 IS INDUCED IN NEURONS AND ASTROCYTES INFECTED WITH HERPES SIMPLEX VIRUS TYPE 1

Initiation of the apoptotic cascade resulting in activation of caspases, which constitutes a central event in the apoptotic process, can be triggered in neuronal cells by a toxic insult, such as exposition to amyloid-β (Aβ) peptides [24]. In fact, neuronal damage and apoptosis induced by Aβ is dependent on the presence of tau [25], which constitutes a caspase substrate. Cleavage of tau by caspase results in a molecule that is more toxic than full-length tau [26] and is more prone to assemble into filaments [27–29], providing a mechanism by which tangle formation may be enhanced or possibly stabilized [30]. In addition, phosphorylation, conformational changes, and cleavage of tau protein are important events that lead to the pathological state of tau protein observed at early stages in neurodegenerative pathologies such as AD. Although the chronology of these changes is still under investigation, the role of caspase-3 in the cleavage of tau at D^{421} is irrefutable [28–31]. In addition, another protein, transactivation response DNA-binding protein 43 (TDP-43), that is cleaved by caspase-3 has been associated to neurodegenerative diseases as AD, Parkinson's disease, and Pick's disease [32–34]. These data suggest that a common mechanism involving cleavage of proteins by caspase-3 is associated with different neurological pathologies.

In a recent study, Lerchundi et al. [35] demonstrated that tau processing at D^{421} also occurs in neurons and astrocytes during HSV-1 infection. In neuronal and astrocytes cultures, tau hyperphosphorylation correlated with caspase-3 activation during HSV-1 infection (Fig. 2), in a similar way to those described in neurodegenerative diseases [29–31]. The dependency of tau cleavage on caspase-3 activation was clearly established using Z-VAD-FMK (fluoromethyl ketone (FMK)-derivatized peptides), an irreversible general caspase inhibitor, before HSV-1 infection, which caused simultaneously the inhibition of caspase-3 and almost undetectable levels of tau cleavage. Interestingly, the effects observed were independent of viral replication since previous treatment of the cells with acyclovir did neither alter tau processing nor caspase-3 activation at 4 hpi. Therefore, these changes were most probably caused by the activation of apoptotic signaling pathways at early time of HSV-1 infection triggered by components (i.e., proteins) of the original viral particles. Accordingly, it was previously demonstrated that in primary hippocampal neuronal cultures, HSV-1 activates the apoptotic JNK (c-Jun N-terminal kinases) pathways independently of viral replication at an early time post-infection [36]. Furthermore, synthetic peptides based on HSV-1 gH protein sequence were tested for MAPK (mitogen-activated protein kinase) cascade activation, showing that restricted domains of HSV-1 gH protein specifically and rapidly activate the JNK pathway [37].

The results presented herein reinforce the idea that besides the pro-apoptotic effects observed in neurons and astrocytes after *in vitro* acute HSV-1 infection, early neurodegenerative events could also be associated to recurrent HSV-1 reactivation in the human brain.

INFLAMMATORY AND NEURODEGENERATION MARKERS DURING ASYMPTOMATIC HSV-1 REACTIVATION

Neuroinflammation triggered by CNS pathogens involve an initial immune innate response characterized by activation of TLRs (*Toll*-like receptors) and other pattern recognition receptors. TLRs activation during neuronal HSV-1 infection has been clearly demonstrated by different groups [38–42]. Using intranasal inoculation of HSV-1 in MyD88 (Myeloid differentiation primary response gene 88, critical adaptor protein for TLR signaling) knockout (KO) mice, Mansur et al. [40] showed that all the animals developed lethal encephalitis after viral inoculation, highlighting the relevance of TLR signaling in the control of viral infection [40, 42]. Previously, Aravalli et al. [39] had shown that TLR2 signaling is important for the production of the proinflammatory

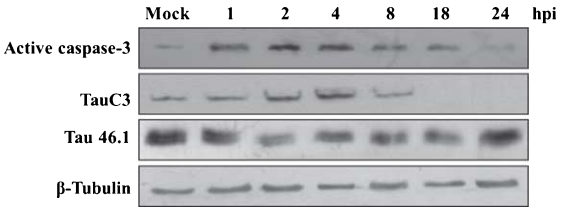

Fig. 2. Caspase-3 activation and tau cleavage induced by HSV-1 infection on neuronal cultures. Western blot analyses show immunodetection with active caspase-3, TauC3, Tau46.1 and Tubulin antibodies in mice primary neurons untreated (Mock) and after 1, 2, 4, 8, 18, and 24 hpi with HSV-1 (moi 10). Blots shown are representative of three separate experiments. Reprinted from [35], *Journal of Alzheimer's Disease*, Volume 23, Lerchundi R, Neira R, Valdivia S, Vio K, Concha MI, Zambrano A, Otth C, Tau cleavage at D421 by caspase-3 is induced in neurons and astrocytes infected with herpes simplex virus type 1, pages 513–520, Copyright (2011), with permission from IOS Press.

cytokines IL-1β, IL-6, and TNF-α, in response to HSV-1 infection. Similarly, Wang et al. [43] showed that TLR2 KO mice had a significantly increased survival rate following intracranial inoculation of HSV-1, compared to wild type and TLR9 KO mice. Likewise, using TLR2, TLR9, and TLR2/9 KO mice, Sørensen et al. [41] concluded that TLR2 and TLR9 synergistically stimulate innate antiviral activities, thereby protecting against HSV infection. All these studies focused on determining the contribution of TLRs and the neuroinflammatory response during productive infection associated to encephalitis.

In an attempt to establish a possible link between neuroinflammation triggered by HSV-1 neuronal reactivation and deterioration of neuronal functions in infected individuals, Martin et al. [8] aimed to evaluate if asymptomatic neuronal reactivation of HSV-1 infection could occur in a mouse model of intranasal inoculation and if a reactivation episode was associated with an increase in early neurodegeneration markers.

In agreement with previous reports, this study showed that TLR2, TLR3, and TLR9 transcripts significantly increased their levels at 15 dpi (days post infection). This increase also occurred for Interferon Regulatory Factor 7 (IRF7) mRNA and phospho-IRF3 protein, both markers associated with activation of TLRs dependent signaling involved in interferon production.

Perhaps the most important finding of this study was the upregulation of TLR4 protein observed in trigeminal ganglia and cortical neurons of some asymptomatic HSV-1-infected mice at 60dpi, which should correspond to the latent phase of infection (Fig. 3A). This upregulation was detected only in animals that showed clear expression of the early viral protein ICP4 at this stage and was accompanied by an increase of p-IRF3 (Fig. 3B), interferon expression and evident astrogliosis in the cortex and trigeminal ganglia, indicative of a persistent neuroinflammatory process, most probably due to viral reactivation from latency.

In a previous study, random testing of 3,200 cerebrospinal fluid (CSF) samples revealed HSV-1 DNA in 26 and HSV-2 DNA in 36 samples from subjects without symptoms of HSV activity [44]. This is an important finding supporting the possibility of frequent asymptomatic reactivation of HSV at neuronal level since detection of viral DNA in the CSF clearly demonstrates that even during clinically silent infections, virus replicates in the CNS [6]. Hua et al. [45] have shown ischemic upregulation of TLR4 in activated microglia of wild type mice, whereas less neuronal damage and activated microglial cells were observed in the ischemic area of the brains of TLR4 KO mice [45]. The authors suggested that activation of TLR4 in microglia contribute to neuronal death, playing a key role in the pathogenesis of cerebral injuries [45, 46]. In addition, Balistreri and colleagues [47] described the involvement of TLR4 in age-related diseases such as neurodegenerative diseases, suggesting a crucial role of molecules of innate immunity in the pathophysiology of these diseases. In addition, treatment of primary murine neuronal cells of TLR4 KO mice with supernatants of amyloid peptide-stimulated microglia demonstrated that TLR4 contributes to amyloid peptide-induced microglial neurotoxicity [48]. Another important finding of this study was a marked upregulation of TLR4 mRNA in the brain of AβPP transgenic mice, and an increased expression of TLR4 in AD brain tissue associated with amyloid plaque deposition, suggesting a role of this key innate immune receptor in neuroinflammatory processes in AD [48].

Furthermore, some authors have suggested that TLR4 has emerged as a new susceptibility marker for AD [49, 50]. Accordingly, reduced TLR4 signaling in response to lipopolysaccharide has been associated with a common mutation of TLR4 gene (Asp299Gly) characterized by declined ability to induce inflammation [49, 51]. Additionally, the Asp299Gly polymorphism has been also associated with a decreased risk of late-onset AD in an Italian population cohort, independent of the susceptibility gene APOE ε4 [52].

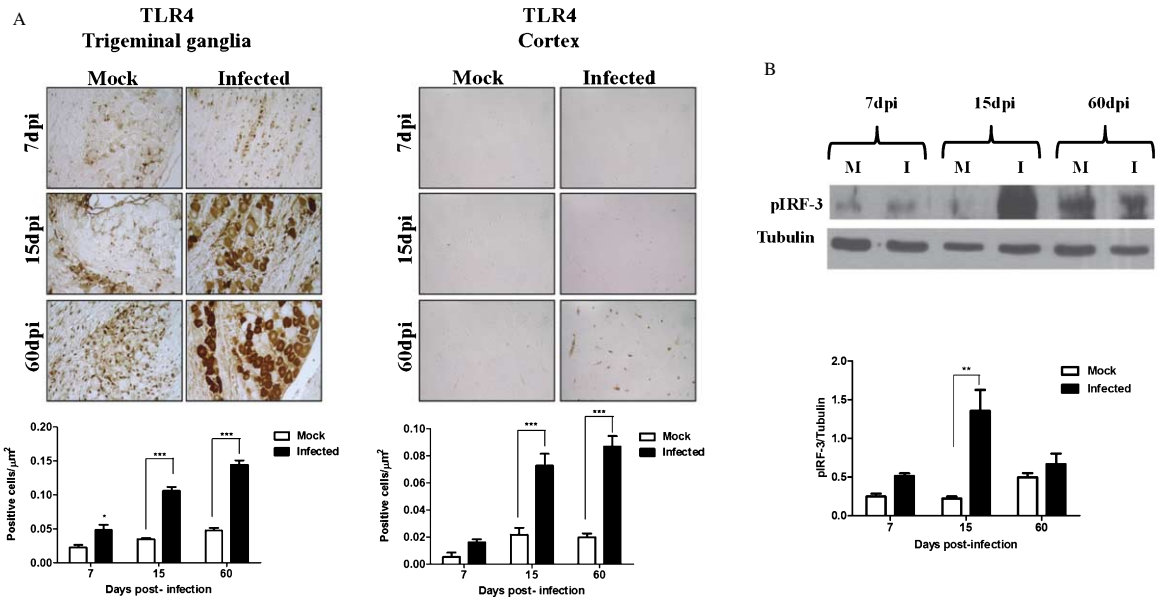

Fig. 3. A) Activation of TLR-dependent pathways in the brain of HSV-1 infected mice. Samples of cortex and trigeminal ganglia of mock and HSV-1 infected mice fixed at 7, 15, and 60 dpi were staining with anti-TLR4 specific antibody. The graphic shows the number of positive cells/μm^2 for TLR4 ($n = 3$). Magnification 100x. $^{***}p < 0.001$; $^{**}p < 0.01$; $p^* < 0.05$. B) Western blot analyses were performed to evaluate the levels of phosphorylated IRF3 (p-IRF3) protein extracted from mock and HSV-1 infected mice brain tissue at 7, 15, and 60 dpi. Levels were normalized using β-tubulin as constitutive protein ($n = 3$). $^{***}p < 0.001$; $^{**}p < 0.01$; $p^* < 0.05$. Reprinted from [8], *Journal of Alzheimer's Disease*, Volume 39, Martin C, Aguila B, Araya P, Vio K, Valdivia S, Zambrano A, Concha MI, Otth C, Inflammatory and neurodegeneration markers during asymptomatic HSV-1 reactivation, pages 849–859, Copyright (2014), with permission from IOS Press.

Coincidently with these findings, Villalba et al. [53] have recently shown increased transcripts encoding TLR2, TLR4, and one of their endogenous ligands, serum amyloid A3 protein (SAA3), in HSV-1-infected astrocytes, suggesting that TLR activation could not only be triggered by the virus but also amplified by this locally produced danger signal [53]. As SAA3 corresponds to an acute phase protein, induced expression of SAA3 transcript has also been demonstrated following exposure to different stimuli, such as oropharyngeal administration of lipopolysaccharide or during cerebral ischemia in mice [54, 55]. Therefore, a possible hypothesis is that the local induction of SAA3 by different triggers could contribute to develop chronic neuroinflammation processes in individuals where HSV-1 has already established latency in the CNS.

The authors show, for the first time, increased levels of early neurodegenerative markers such as hyperphosphorylated and cleaved tau protein (p-tau and TauC3 markers, respectively) during *in vivo* HSV-1 neuronal infection in mice. This constitutes a relevant finding since recent studies indicate that neuronal dysfunction precedes the formation of tau insoluble fibrillary deposits, suggesting that earlier tau dysfunction could be sufficient to cause neurotoxic effects and neurodegeneration [56]. Additional support for this idea comes from previous evidence showing severe spatial memory deficits and chronic lesions derived from decreased brain volume, neuronal loss, activated astrocytes, and glial scar formation to severe atrophy in herpes simplex encephalitis (HSE) surviving animals during latent infection [57, 58].

All these findings contribute to support the hypothesis that the presence of HSV-1 in the CNS could promote chronic neuroinflammation by recurrent reactivation episodes, which trigger TLRs activation and as a result could constitute a risk factor of neurodegenerative processes.

MODULATION OF THE AMPK/SIRT1 AXIS DURING NEURONAL INFECTION BY HERPES SIMPLEX VIRUS TYPE 1

Previous reports have shown that HSV-1 inhibits apoptosis during early infection, but is pro-apoptotic during late productive infection [59–62], suggesting a time course modulation of apoptosis during HSV-1 infection. In fact, the existence of a critical

relationship between metabolic sensing pathways and innate immune responses to different pathogens has been proposed [63]. In this context, recent studies have reported that different viruses target the host-cell metabolic machinery during cell infection, suggesting a key role of metabolic function during viral infection [64, 65]. Neurons require high levels of ATP in order to sustain various neuronal processes such as firing of action potentials, neurotransmission, and ion homeostasis. Interestingly, it has been proposed that mitochondria may exert a crucial role in the pathogenesis of inflammatory and neurodegenerative disorders [66], and play a central role in the primary host defense mechanisms against viral infection, where a number of novel viral and mitochondrial proteins are involved in these processes [67]. Conversely, little is known about the possible mechanisms that HSV-1 utilizes to highjack the neuronal metabolic pathways, counteract antiviral, and stress sensing signaling pathways.

Sirtuin 1 deacetylase (Sirt1) modulates fundamental mechanisms in aging-related neurodegenerative diseases, including protein aggregation, stress responses, mitochondrial homeostasis, and inflammatory processes. On the other hand, AMP-activated kinase (AMPK) is a central regulator of cellular energy that regulates a number of cellular pathways that can influence viral replication, including protein and lipid biosynthesis [68]. In addition, Sirt1 and the fuel-sensing enzyme AMPK have been involved in neuroprotection and both regulate each other and share many common target molecules, including the pro-apoptotic protein p53 and the master regulator of mitochondrial biogenesis peroxisome-proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α).

Our study [69] reported that HSV-1 modulates AMPK/Sirt1 axis during the course of *in vitro* neuronal infection. The results showing a clear reduction of activated AMPK (p-AMPK), reduced Sirt1 activity and increased levels of acetylated p53 at 2hpi suggest that at early times post infection HSV-1 inhibits the AMPK/Sirt1 axis in neurons, which would favor activation of p53-dependent apoptotic pathways (Fig. 4). In fact, in a previous study we showed that in HSV-1 infected neurons maximal activation of caspase-3 was observed at 4 hpi and decreased thereafter [35]. An early increase in acetylated p53 has also been described in mouse embryonic fibroblast infected with HSV-1 and have been shown to be essential for p53-mediated antiviral activity [70]. In neurons, these effects were observed before viral gene expression and perhaps could be triggered by tegumental proteins such as UL13, a promiscuous serine/threonine protein kinase, which has been reported to activate apoptosis and has the ability to inhibit the antiviral type 1 interferon response [71]. The early induction of programmed cell death in infected cells constitutes an effective antiviral host mechanism to restrict viral spread within an organism. As a countermeasure, viruses have evolved numerous strategies to interfere with the induction or execution of apoptosis. Slowly replicating viruses such as HSV-1 are particularly dependent on sustained cell viability. In fact, another tegumental protein kinase, US3, mediates antiapoptotic activity through phosphorylation and regulation of pro-apoptotic Bcl2 (B-cell lymphoma 2) family members [72, 73]. However, several recent studies showing that caspase-3 activity triggers the replication of Kaposi's sarcoma-associated herpesvirus (KSHV) and HSV-1, suggest that a caspase-3-dependent mechanism of viral replication is a common feature in the family of herpesvirus [74, 75].

Another important aspect to consider about the initial inhibition of the AMPK pathway caused by HSV-1 infection is that this strategy would benefit viral replication because the biosynthesis of viral proteins and lipids would not be shut-down [65, 76]. However, the initial inhibition of the AMPK/Sirt1 axis was gradually reversed starting at 4 hpi, evidenced by the marked increase in Sirt1 protein and the reduction in acetylated p53. Considering that, HSV-1 depends on neuronal survival for its persistence in the organism in latent state; the inhibition of AMPK/Sirt1 axis, should be neutralized by the virus to restore energy homeostasis (Fig. 4). In fact, maximal activation of Sirt1 and AMPK was achieved between 8 and 18 hpi, coinciding with viral protein expression and also with the increase of PGC-1α protein and mitochondrial transcription factor A (TFAM) transcript levels. In contrast, a different behavior was observed in U251 glioma cells in which HSV-1 does not establish latency. In these cells, the inhibition of AMPK after HSV-1 infection persists and is intensified after 2 hpi [76]. AMPK activation inhibits protein translation by inhibiting mTORC1 (mammalian Target of Rapamycin Complex 1) activity and through inactivation of the translation factor eEF2 (eukaryotic elongation factor-2). In addition, activation of AMPK through stress or low energy conditions inhibits fatty acid synthesis through inactivation of acetil-CoA carboxilasa (ACC). The AMPK/mTOR pathway also regulates autophagy, which can destroy

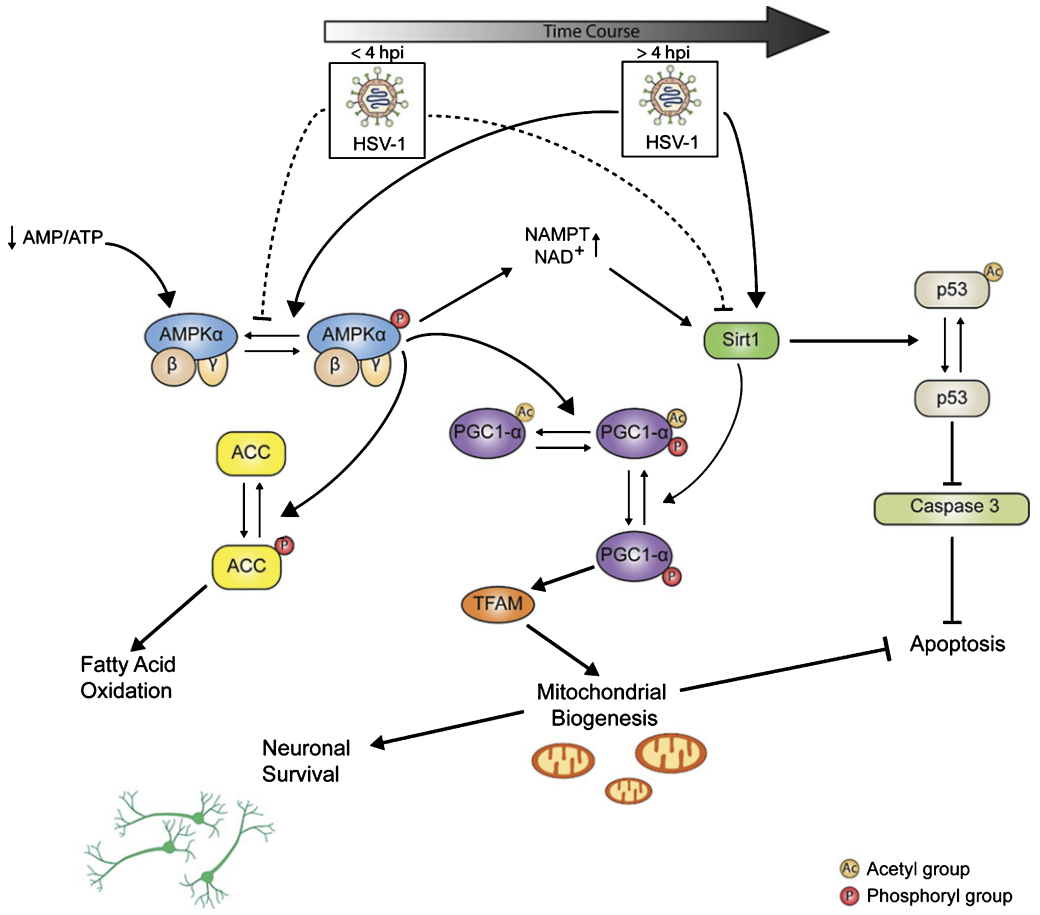

Fig. 4. Proposed model for the modulation of AMPK/Sirt1 axis during HSV-1 neuronal infection. HSV-1 infection induced an early decrease of p-AMPK levels and increased acetylation of p53, but after Sirt1 upregulation and activation (4 hpi), a gradual increase in p-AMPK and a marked reduction in ac-p53 was observed. In parallel, upregulation of PGC1 and TFAM was detected suggesting the stimulation of mitochondrial biogenesis. These results suggest that HSV-1 infection affects normal neuronal metabolism modulating the AMPK/Sirt1 axis to favor viral propagation. Reprinted from [69], *Journal of Alzheimer's Disease*, Volume 42, Martin C, Leyton L, Arancibia Y, Cuevas A, Zambrano A, Concha MI, Otth C (2014) Modulation of the AMPK/Sirt1 axis during neuronal infection by herpes simplex virus type 1, pages 301–312, Copyright (2014), with permission from IOS Press.

cytosolic pathogens. While the evasion of autophagy by pathogens has been demonstrated, recent work suggests that both the AMPK/mTOR pathway and autophagy itself can provide intracellular metabolites that support intracellular pathogen replication. Recently it was also shown that activation of AMPK is critical for the replication of human cytomegalovirus, a member of the beta herpesvirus family [64]; and is important for facilitating the entry of vaccinia and Ebola virus through its effects on macropinocytosis [77]. In contrast, AMPK could efficiently restrict infection by the Rift Valley Fever Virus and other viruses by inhibiting fatty acid metabolism [65]. Finally, hepatitis C virus was found to inhibit AMPK activity by promoting its dephosphorylation, which is required for hepatitis C virus replication [68].

In addition to the modulation of Sirt1 and AMPK activity by HSV-1, an intriguing observation of our study was the marked redistribution of both proteins and also of acetylated p53 from the nucleus to cytoplasmic foci. This relocalization was evident between 4 and 8 hpi. Recent studies have described nucleocytoplasmic shuttling of Sirt1 in response to oxidative stress [78–81] and also a Sirt1 subcellular redistribution has been described in AD neurons [82]. Since Sirt1 has both nuclear and cytoplasmic targets, this redistribution should have important effects on neuronal functionality. Although Sirt1 and PGC1 are considered important inducers of mitochondrial biogenesis through regulation of transcription of nucleus-encoded mitochondrial genes, it has been recently demonstrated that PGC-1α

and Sirt1 are also present inside mitochondria, in close proximity to mitochondrial DNA, forming a multiprotein complex with TFAM, suggesting their possible involvement in regulation of mitochondrial biogenesis and metabolism [76]. Similarly, the nuclear transcription factor p53, which activates genes involved in apoptosis, cell cycle regulation, and numerous other processes, also plays a crucial role in the transcriptional-independent apoptotic pathway, relocating and inducing apoptosis directly at mitochondria, via the interaction with members of the Bcl-2 family [83]. Moreover, Kawaguchi and colleagues demonstrated that C-terminal lysines of p53 are involved in the acetylation-mediated nuclear export and cytoplasmic accumulation of p53 [84]. In addition, cytoplasmic p53 has also been shown to inhibit autophagy [85].

Concerning AMPK, this metabolic sensor is predominantly expressed in neurons and strongly localizes to the nucleus in the mammalian adult brain. Our results indicate that p-AMPK relocates from the nucleus to discrete cytoplasmic foci in infected neurons after 4hpi. In this context, it is interesting to highlight a recent study that showed that AMPK is abnormally activated and accumulates in the cytoplasm of cerebral neurons in different tauopathies, including AD [86]. Whether this over activation of AMPK is neuroprotective or neurotoxic is still a matter of debate. However, AMPK activation has been shown to protect primary neuronal cultures from excitotoxicity and several other insults, including exogenous Aβ treatments [87]. Different treatments with known activators of AMPK, such as resveratrol, have been found to prevent different neurodegenerative mechanisms in cell culture systems or mouse models [86, 88, 89]. Moreover, AMPK is a physiological tau kinase, and its activation decreases mTOR signaling activity stimulating autophagy and promoting lysosomal degradation of Aβ [90].

Taking in consideration the important contribution of the AMPK/Sirt1 axis in the metabolic homeostasis of neuronal cells, the results shown here of the ability of HSV-1 virus to manipulate these pathways, further contribute to envision possible mechanisms involved in pathogen-triggered neurodegeneration processes. Recently Leyton et al. [91] evaluated if natural activators of the AMPK/Sirt1 axis, such as resveratrol and quercetin could reduce viral propagation and/or counteract the effects of neuronal infection. The results obtained in the study support the notion that resveratrol or quercetin treatments reduce HSV-1 production efficiency and protect neurons from damage triggered by viral replication through the activation of AMPK/Sirt1 axis. These findings suggest that these nutraceuticals could be potentially helpful in the prevention of neuronal damage associated with recurrent neuronal HSV-1 reactivations, down-regulating cell signaling necessary for optimal viral replication efficiency. A previous study has suggested that treatment with valacyclovir could be an alternative to prevent neurodegeneration triggered by HSV-1 reactivations [92]. Also, lysine supplementation has been suggested to result in beneficial effects by reducing HSV-1 replication [93]. Nevertheless, this study suggests that resveratrol and quercetin could be especially helpful in immunodepressed patients, which are at higher risk of recurrent HSV-1 reactivation and accordingly more exposed to neuronal damage triggered by neuronal HSV-1 infection.

CONCLUSIONS

HSV-1 causes a rare but very serious acute, neurological condition called HSE. In the initial stages of this disease, necrosis of the frontal and/or temporal lobes occurs and viral antigen can also be detected in the hippocampus, amygdala, cingulate gyri, and olfactory tracts. All of these regions are affected in AD. Thus, HSV-1 is able to selectively destroy the very same cells that are affected in AD [2]. However, one major problem with the proposed role of HSV-1 in AD is that HSE causes a large amount of damage in a relatively short period of time whereas the damage seen in AD is progressive, accumulating over several years. Thus, if HSV-1 were to cause AD, it would have to produce a milder and probably recurrent disease. Interestingly, a mild form of HSE has been documented [94], and there have been a number of case reports of recurrent HSE. It has been suggested that AD might be caused by episodes of mild HSE [95] and as survivors of both mild HSE and full blown HSE experience memory loss; clearly, HSV-1 can cause this loss, the main neurological symptom of AD [96].

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Anti-Viral Properties of Amyloid-β Peptides

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Abstract. Amyloid-β (Aβ) peptides generated by the amyloidogenic pathway of amyloid-β protein precursor processing contribute significantly to neurodegeneration characteristic of Alzheimer's disease (AD). The involvement of Aβ peptides in the etiology of AD remains a subject of debate. Data published in the last 6 years by three different groups have added a new twist by revealing that Aβ peptides could act as antimicrobial peptides (AMP) in *in vitro* assays against some common and clinically relevant microorganisms, inhibit replication of seasonal and pandemic strains of influenza A and HSV-1 virus. These observations are of significance with respect to the notion that pathogens may be important contributors to the development of AD, particularly in the case of herpes simplex virus (HSV) infection, which often resides in the same cerebral sites where AD arises. Here, we review the data that support the interpretation that Aβ peptides behave as AMP, with an emphasis on studies concerning HSV-1 and a putative molecular mechanism that suggests that interactions between Aβ peptides and the HSV-1 fusogenic protein gB lead to impairment of HSV-1 infectivity by preventing the virus from fusing with the plasma membrane. A number of avenues for future research are suggested.

Keywords: Alzheimer's disease, amyloid-beta peptides, antimicrobial peptides, antiviral activity, cocultures, glycoprotein B, herpes simplex virus, influenza virus, membrane proximal region

INTRODUCTION

In 1907, Alois Alzheimer described a female patient who presented unusual symptoms of dementia [1]. Postmortem examination of the brain of this patient with extensive cognitive deterioration revealed the presence of cortical atrophy associated with senile plaques and neurofibrillary tangles. This single historical case description has had far-reaching consequences in what has become known as Alzheimer's disease (AD). AD is now recognized as the most common form of dementia in the world [2, 3]. It is a progressive neurodegenerative disorder that is characterized by irreversible neuronal degeneration in specific regions of the brain, especially the neocortex and the hippocampus, which is the seat of memory [4, 5]. The clinical manifestations of AD are an initial decline in short term memory that progresses over the years to complete loss accompanied by impaired language skills, alterations of cognitive functions including rational judgment and decision making, a loss of self-autonomy and, in a large num-

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ber of cases, uncontrolled and aggressive behavioral disturbances [6–8]. AD has become a growing public health concern. Surveys estimate that AD currently affects over 47 million patients worldwide and projections for the next decades are staggering. According to conservative models of AD epidemiology, progression from early diagnosis to full-blown disease is projected to affect over 65 million individuals in 2030 and more than 131.5 millions by the year 2050 [9, 10]. Collateral costs put a severe burden not only on the affected individuals but also on families, professional caretakers, specialized home care institutions and society in general. From a standpoint of monetary costs, global estimates of the financial expenditure in 2010 exceeded 600 billion US dollars [11] and will likely reach trillions of dollars if current projections are accurate. This paramount medical and social problem is further compounded by the general increase in life expectancy and the fact that aging is the primary risk factor, at least in the age-related form of AD [12–16]. Despite extensive efforts documented by more than 116,854 references to AD currently archived in PubMed® and 7,076 references to its prevention at the moment of writing, there is still no efficient treatment to halt progression of the disease, let alone a cure for it [17–19]. AD is a highly complex and debilitating disease that manifests as an overall deterioration of the human condition and brings irrevocable earlier death, in nearly all cases [20]. Clinical interventions have targeted general and neuropsychiatric symptoms by using cholinergic inhibitors, *N*-methyl-*D*-aspartate (NMDA) receptor antagonists and behavior control drugs, inhibition of production of fragments of amyloid-β protein precursor (AβPP) processing [21], anti-inflammatory drugs [22, 23], as well as medications that target metabolic aberration products associated with AD [6, 24, 25]. These multi-targeted approaches are reflected by the fact that the underlying causes of AD have not been clearly established, making efficient treatment highly difficult [26]. Aside from the obvious relationship to aging, particularly in the case of the late onset form of the disease, several possibilities have been put forward as risk-associated conditions. These have been recently reviewed [27]. They include environmental factors [28], head injury [26, 29], malnutrition [30, 31], structural changes in the vasculature [32–34], alterations of the cholinergic and cortico-cortical pathways [35, 36], genetic factors [37–39], alterations in immune functions [40–42], mitochondrial dysfunction [2, 43], altered blood-brain barrier [44], pathogen and virus infections [45–50], and local and systemic inflammation [51–54].

EARLY AND LATE FORMS OF AD

There are two clinical forms of AD, according to their characteristic pathogenesis and the time of onset. One form of the disease is referred to as the early onset AD (EOAD) and it corresponds to the genetic and familial form of the disease. The late onset AD (LOAD) is the sporadic manifestation of the disease that generally occurs after the age of 65. However, both forms are characterized by similar pathological changes namely, irreversible neuronal loss and deposits of cortical senile plaques and formation of neurofibrillary tangles in the brain of affected patients [20, 55]. EOAD represents 5% to 10% of documented AD cases whereas LOAD accounts for the remaining number of cases. Three genes involved in amyloid-β protein precursor (AβPP) metabolism are considered the main risk factors for EOAD: AβPP itself and AβPP-processing proteases presenilin 1 (PSEN1) and presenilin 2 (PSEN2) [56]. AβPP is a single-pass type 1 transplasma membrane protein [57] that is expressed in the central nervous system (CNS) as well as most somatic tissues [58, 59]. Although the physiological role of AβPP is still not clear, the bulk of data collected so far suggests that AβPP may be involved as a trophic factor to provide help for neurite outgrowth and synaptogenesis [60, 61], especially in the developing brain and to play a role in neuronal signaling functions [62, 63]. Expression of AβPP is influenced by trauma to the brain. For instance, its expression is upregulated in AD [64] and following brain injury, in which case it may be essential to participate to restore synaptic function [65]. At least 25 pathological mutations in AβPP have been associated with EOAD [2, 66].

So far, no gene has been identified as the cause of LOAD. However, environmental factors, family history, diabetes mellitus, educational status, hypertension, hypercholesterolemia, brain infection, and head trauma have been suggested as risk factors that may contribute to LOAD [15, 16, 67–69]. Furthermore, mutations/variants of a number of genes have shown strong association as risk factors of LOAD. Among these, inheritance of the apolipoprotein E ε4 (APOE ε4) allele appears to be the most prominent candidate [56, 70–73]. However, genome-wide association studies have also identified medium-to-low risk gene products such as triggering

receptor expressed on myeloid cells 2 (TREM2), an innate immune receptor expressed on a variety of cells including microglia [74, 75], phospholipase D3 (PLD3), a widely expressed phospholipase for triglyceride metabolism, as well as a large number of gene products associated with immune response, cell physiology and epigenetics [25], and several other candidate genes [56, 76].

PROCESSING OF THE AMYLOID-β PROTEIN PRECURSOR

Proteolytic fragments generated from extracellular and intracellular portions of the molecule

The gene coding for AβPP is located on chromosome 21 and is interspersed with 18 exons. Alternative splicing of gene transcripts results in several isoforms of AβPP. The AβPP695 isoform lacks the gene sequence from exons 7 and 8. This is the isoform preferentially expressed in neurons [77, 78]. AβPP is proteolytically processed by two competing pathways (Fig. 1). One pathway gives rise to amyloid-β (Aβ) peptides and other fragments and is called the amyloidogenic pathway. The other pathway does not generate Aβ peptides but fragments of a different structure and is called the non-amyloidogenic pathway. In the non-amyloidogenic pathway, AβPP is cleaved by α-secretases (ADAM proteases/TACE) to generate a large extracellular soluble secreted fragment (sAβPPα) and the plasma membrane-associated αAβPP-CTF fragment of 83 amino acid residues (C83). C83 is further cleaved by γ-secretase [79] to release a P3 fragment and the AβPP intracellular domain (AICD). Whereas the physiological role of αAβPP-CTF has not been clearly established, AICD may translocate to the nucleus and play a role in the transcription of AβPP [80, 81]. In the case of the amyloidogenic pathway, AβPP is internalized into endocytic compartments (Fig. 1B) where it is cleaved into two fragments termed sAβPPβ and AβPP-C99, as the result of the proteolytic activity of the β-secretase BACE. AβPP-99 is then cleaved by a γ-secretase complex comprising presenilin 1 to generate AICD and Aβ peptides, which are secreted [82]. The major isoforms of Aβ peptides are composed respectively of 38 ($A\beta_{38}$, <20%), 40 ($A\beta_{40}$, <80%), and 42 ($A\beta_{42}$, ≈ 10%) amino acid residues [83]. Although similar in molecular size, $A\beta_{40}$ and $A\beta_{42}$ differ in their physical properties. For instance, hydrophobicity and propensity to oligomerize into a fibrillar form and cytotoxicity are mostly associated with the $A\beta_{42}$ peptide [84–87].

Aβ peptides and microtubule-associated tau protein solubility is the key to a healthy brain

Extracellular formation of proteolysis-resistant insoluble fibrils of Aβ peptides that deposit in senile plaques and, intracellular neurofibrillary tangles resulting from hyperphosphorylation of the microtubule-associated protein tau, are the neuropathological hallmarks of AD [6, 86–90]. Tau is an essential component of microtubules, which are one of the fundamental elements of the cytoskeleton involved in anterograde and retrograde transport of vesicles, space distribution of mitochondria, and chromosome partitioning during cell division. In AD, tau can form insoluble fibrils that deposit inside the cell [91]. Human tau is encoded by the microtubule-associated protein tau gene, MAPT, that comprises 16 exons and which gives rise to 6 isoforms [92]. The longest form of tau comprises 441 amino acid residues and the shortest, 352. In the brain, tau is mainly found in neurons but is also present at low levels in glia1 cells. Tau can undergo a large number of post-transcriptional modifications that include phosphorylation, glycosylation, deamidation, and acetylation, among others [93]. Tau is a highly hydrophilic protein since the longest form contains 80 hydrophilic amino acid residues and 114 polar (acidic and basic) amino acid residues. Therefore, tau is a soluble protein and it is expected that phosphorylation would favor its water solubility. In this respect, physiological tau is phosphorylated and this is an essential modification required for its functions, namely its associative role with microtubules and microtubule-associated proteins [94]. However, it is abnormally hyperphosphorylated in the AD brain [95–99]. This observation has been taken as evidence for its neurotoxicity [91] and shown to be a reliable feature of AD. It has been suggested that hyperphosphorylated tau may be the major culprit in the pathology of AD [91]. It is still unclear how hyperphosphorylated tau assembles into intracellular (and extracellular) insoluble fibrillary structures and how this behavior relates to the mechanism of its neurotoxicity.

Aβ are normal products of AβPP processing, although the mechanism that favors the non-amyloidogenic over the amyloidogenic pathway remains under investigation. $A\beta_{40}$ and $A\beta_{42}$ peptides differ by only two amino acid residues at the

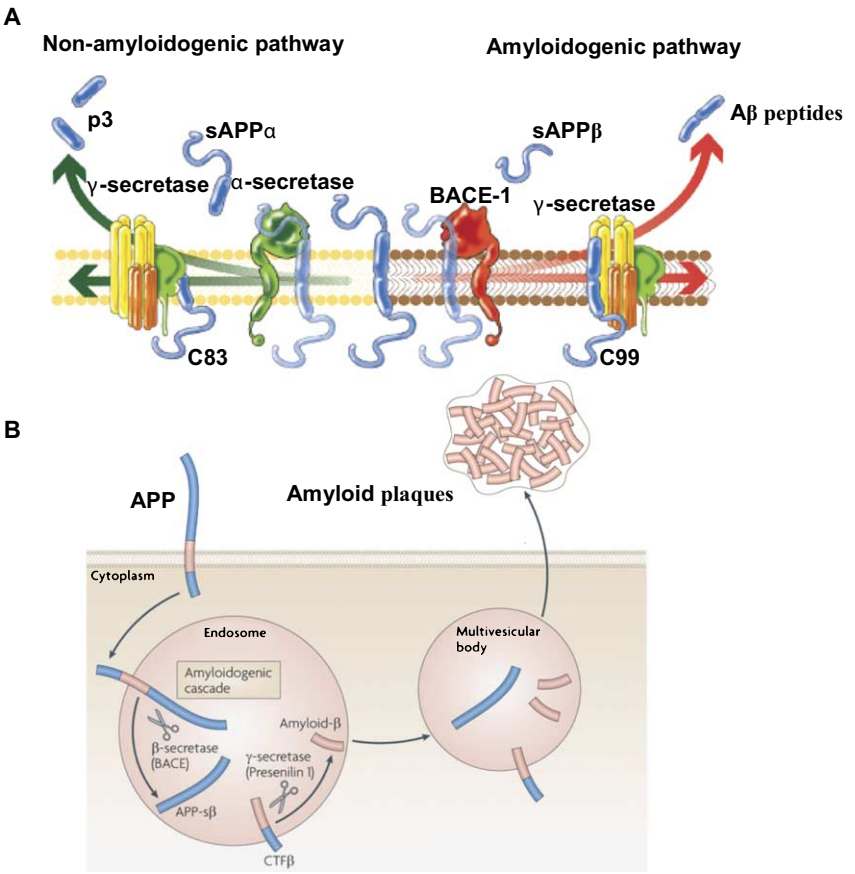

Fig. 1. Diagrams depicting the processing of amyloid-β precursor protein (APP) by the non-amyloidogenic and amyloidogenic pathways in neuronal cells. A) Illustration of the various proteases involved in the generation of fragments from APP processing. Processing enzymes are α-secretase (green), β-secretase (BACE-1, red) and γ-secretase (yellow). Proteolytic fragments are also represented, including Aβ peptides. (Reproduced from [121], Zolezzi et al. (2014) *Front Aging Neurosci* **6**, 176). B) Illustration of the endocytic steps that lead to the production and secretion of Aβ peptides by way of the amyloidogenic pathway and, senile plaque formation (Reproduced from [205], Rivest (2009) *Nat Rev Immunol* **9**, 429-439, with permission from Nature Publishing Group).

C-terminal but they display intrinsic physical differences. For instance, $A\beta_{42}$ is more hydrophobic and more cytotoxic than its $A\beta_{40}$ counterpart [85, 100, 101]. These structure-related features that may be due to its properties to be more prone to aggregate than $A\beta_{40}$, are due to the presence of the additional two aliphatic (Ile and Ala) amino acid residues at the C-terminal. Aβ can assemble in oligomeric channel structures, as shown in model plasma membranes [87, 102, 103], a property that would confer cell toxicity. Although Aβ are one of the two hallmarks of AD, their production under normal conditions suggests that they play an important physiological role. For instance, $A\beta_{40}$ and $A\beta_{42}$ are present in the cerebrospinal fluid at concentrations of approximately 1,500 pM and 200 pM, respectively, and at concentrations are 60 pM and 20 pM in plasma, respectively [104]. Although Aβ have been considered a harmful byproduct of AβPP processing, they also play a beneficial role in the regulation of memory in humans [105], neurotrophic effect in differentiating neurons [106], neuroprotection, growth and survival in *in vitro* cultures of rat cortical neurons [107, 108], as well as synaptic plasticity [109]. Of considerable significance (to be discussed below), Aβ peptides may also play a role as antimicrobial agents in the brain. However, the notoriety of Aβ resides in their association with AD. In fact, accumulation of deposits of Aβ in a filamentous (insoluble) form is associated with neuronal degeneration and cortical atrophy [6, 25, 26, 90, 110]. This finding has served as the basis to the *amyloid cascade hypothesis* of AD. This hypothesis has been the predominant framework for research in AD since it was initially put forward [111]. In

essence, the hypothesis postulates that deposits of Aβ in senile plaques is the cause of AD and "that neurofibrillary tangles, cell loss, vascular damage and dementia follow as a direct result of this deposition". However, data accumulated over the years have shed doubts on this hypothesis as the major, if not the only cause of AD. The controversies have been the subject of recent reviews arguing against the hypothesis [112] or favoring its modification as an essential component of the complex AD picture [17, 109, 113, 114]. Currently, it is likely that deposits of Aβ are initiators of a complex pathogenic cascade that involves immune/inflammatory responses [14, 51, 53, 86, 115–118], tau aggregation [119], neuronal cell death, and neurodegeneration. The mechanism of Aβ accumulation in LOAD is not fully understood but appears to be caused by overproduction or by a defect in clearance and degradation by microglial cells [120–123], or both. In addition, the defect in Aβ clearance is further aggravated by age-related immune changes such as immunosenescence and inflamm-aging [124–128].

INADEQUATE CLEARING OF Aβ PEPTIDES: A COMPONENT OF THE INITIATOR PROCESS THAT LEADS TO AD?

Microglial cells fulfill immunomodulatory functions in the brain and are recognized as resident macrophages [123]. These cells migrate to the site of insult in response to invading pathogens and brain injury [129]. The nature of these injuries may be brain trauma [130], damaged neurons [131], the presence of amyloid plaques, and Aβ aggregates [14]. Microglial cells are involved in Aβ clearance [40, 132]. Therefore, they play a determining role under normal conditions but their activity may be reduced under pathological conditions [14], notably when there has been excess production of Aβ. This situation may contribute to progression to AD and may be part of the onset of the disease. Microglial cells express several plasma membrane receptors for Aβ. These are scavenger receptors, receptor for advanced glycation endproducts, CD36, Fc receptors, and toll-like receptor [133]. Occupation of these receptors induces the switch to a neurotoxic state and the production of the inflammatory cytokines interleukin 1β and 6 (IL-1β, IL-6) and tumor necrosis factor α as well as reactive oxygen species and nitric oxide [14, 69, 134, 135]. These cytokines are responsible for the neuroinflammation associated with AD [51, 52, 136–140], including apoptosis and necrosis of damaged neurons [141]. Furthermore, these inflammatory cytokines may negatively influence Aβ clearance, further increasing their accumulation as a result of alteration of Aβ receptor function [142]. In addition, this pathological situation is aggravated by the increased permeability of the blood-brain barrier (BBB) in aging and AD [14, 139, 143, 144]. Alteration of the BBB allows increased communication between the brain and the periphery, thus establishing a pernicious cycle of sustained immunoinflammatory status. This possibility has been recently reviewed and discussed at length in a recent paper by Goldeck et al. [14]. In essence, initial insult to the brain (trauma, pathogenic infection, Aβ deposits) would trigger activation of microglia and astrocytes and increase the production of Aβ and inflammatory cytokines. On the one hand, the chronic load of Aβ would overwhelm the phagocytic activity of microglia, favoring their deposit as amyloid plaques and the generation of neurofibrillary tangles of hyperphosphorylated tau. On the other hand, the release of brain inflammatory mediators to the periphery through damaged BBB would trigger the peripheral inflammatory response of innate and adaptive immunity. The peripheral inflammatory response in turn would favor the release of pro-inflammatory cytokines and other mediators that would reach the brain through altered BBB. This irreversible pernicious cycle of interconnections between inflammatory processes in the brain and their transmission to the periphery, and back to the brain, would further increase neuronal damages and cripple synaptic communications leading to irreversible progression to AD. This pathological situation may become amplified with age due to immunosenescence/inflamm-aging (Fig. 2).

BRAIN INFECTION BY VIRUSES OF THE *HERPESVIRIDAE* FAMILY

The case of herpesviridae viruses: Herpes simplex virus-1 (HSV-1) and cytomegalovirus (CMV)

The realization that infectious organisms are involved in the etiology of AD has been gaining momentum in the scientific community. For instance, two editorials signed by several investigators in the field have made convincing arguments in favor of this hypothesis [145, 146]. Various infectious agents have been associated with cognitive decline and the possible onset and progression of AD (Table 1).

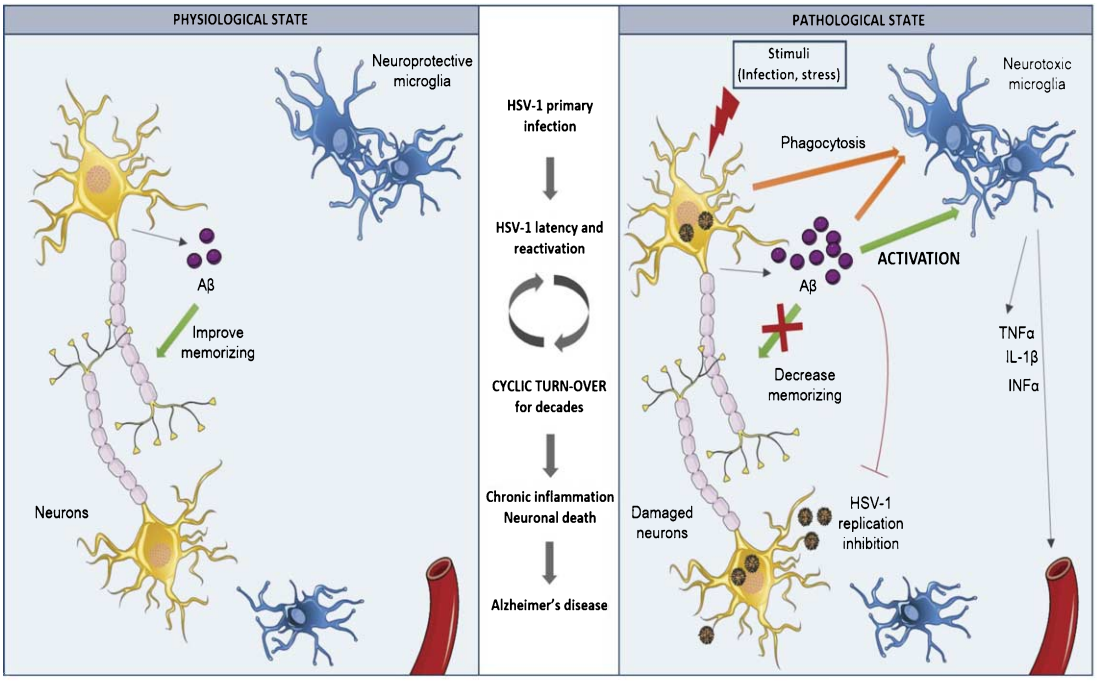

Fig. 2. Model depicting the influence of Aβ in response to infection in brain inflammation and AD. Aβ are normally produced by neurons under homeostatic conditions. A balance between Aβ production and clearance is regulated by microglia-dependent phagocytosis (physiological state). Brain trauma such as infections and other insults would trigger a protective mechanism involving Aβ production. For instance, HSV reactivation would trigger increased Aβ production, activation of microglia and release of pro-inflammatory cytokines (pathological state) that would sustain microglia activation and initiate a pernicious circle of inflammatory responses. In addition, pro-inflammatory cytokines can cross the altered blood brain barrier, initiating systemic inflammation. Overproduction and accumulation of Aβ would interfere with their physiological functions, induce damages to neurons to which Aβ oligomers will associate in a fibrillar form and generate senile plaques and favor progression to AD. (Cell images were adapted from Servier Medical Art, with permission). (Reproduced from [178], Bourgade et al. (2015) *Biogerontology* **16**, 85-98).

Table 1
Infectious agents reported to be associated with the development of AD

Bacteria		Viruses	Eukaryote Fungi and Protozoans
Non-Spirochetes	Spirochetes		
Helicobacter pylori	*Borrelia burgdorferi*	Herpes simplex virus 1	*Candida glabrata*
Chlamydophila pneumoniae	*Treponema sp.*	Human Herpes virus 6	*Toxoplasma gondii*
Porphyromonas gingivalis		Cytomegalovirus	
		Epstein-Barr virus	

Modified from [54].

A number of reviews have summarized the association between bacteria (particularly Spirochetes), viruses, fungi, and protozoans, and the fact that these agents can be detected in the brain of AD patients, specifically in senile plaques [26, 47–49, 54, 147, 148]. With respect to viral infections, it has been suggested that they are a contributing factor to AD [26, 45, 48, 50, 145, 146]. In fact, the hypothesis of microbial agents as a possible cause of AD dates back to close to 25 years [147–154]. In 1998, Balin et al. [151] suggested that infection with Chlamydia pneumoniae (now re-named Chlamydophila pneumoniae) was a high risk factor in the development of AD. Furthermore, Itzhaki et al. [46] suggested that infection with HSV-1, when it was present in the brain of carriers of the APOE ε4 alleles, was a risk factor for the development of AD. This hypothesis was in agreement with previous suggestions of the involvement of viruses in neurodegeneration [155] and, HSV-1 in AD [156]. HSV-1 is frequently found in amyloid plaques [48, 157, 158]. In addition, other members of the herpes virus family, namely HSV-2, CMV, and HHV-6, have also been detected in the brain of AD patients [159] or have been associated with its pathogenesis [157].

HSV-1 is a virus, which infects most humans early (before 10 years of age) in life in settings with low socio-economic conditions, including the "developed world" prior to 1970. However, HSV-1 infection is now acquired later, including increased sexual transmission [160]. The virus is able to remain latent during the whole life of the infected individuals [161]. HSV-1 is capable of escaping immune recognition by remaining hidden in the trigeminal ganglions but can be reactivated under conditions of immunodeficiency or stress. Under these conditions, HSV-1 can re-infect the host [162] and colonize the hippocampus and fronto-temporal lobes [154]. Reactivation of HSV-1 can have minor effects but can, in some cases, trigger lethal herpetic encephalitis that also occurs in the same areas of the brain as those affected in AD (hippocampus and frontal and temporal cortical lobes) [163–165].

In the case of CMV, immunodetection has been used to show its association with AD [166]. However, conclusions of these findings have led to a debate [167, 168] whether the association with AD was supported by sufficient evidence, leaving the question unanswered. Recently, it has been reported that CMV behaves as a cytokine-related promoter of inflammation in relationship to AD [169].

Aβ AS ANTIMICROBIAL PEPTIDES (AMP) AGAINST MICROORGANISMS

Aβ peptides can self-assemble into Aβ structures, a common feature of misfolding for pathological proteins and can form channel structures in cellular plasma membranes [87, 102, 103, 170, 171], a property that resembles that of channel-forming toxins [172]. Consequently, the formation of leaky channels or pores induces lysis of the targeted organism, leading to cell death. The fact that the cytotoxicity of Aβ was related to its aggregated form and much less or none to its monomer or fibrillar form [173], led to the possibility of a parallel between their channel-forming property and their activity as AMP. This possibility was investigated by Soscia et al. [174]. In this groundbreaking publication, the authors compared the AMP activity of $A\beta_{40}$ and $A\beta_{42}$ peptides to that of LL-37, the only human member of the cathelicidin AMP family [175, 176]. Results showed that the Aβ peptides displayed AMP activity against eight of twelve clinically relevant infectious microorganisms (Table 2). Furthermore, colony-forming unit assays revealed that Aβ possessed AMP potency equivalent or even superior to LL-37 and that this activity was reduced by neutralizing antibody depletion. Of significance, the authors reported that Aβ-containing brain homogenates from AD patients displayed AMP activity against *Candida albicans,* in contrast to homogenates from AD-free subjects. On the basis of Aβ being localized at the membrane of *Enterococcus* faecalis, for example, the authors suggested that this observation was consistent with the interpretation that Aβ peptides associated with the bacterial membrane. The bulk of these observations led the authors to conclude that, "Our findings suggest Aβ is a hitherto unrecognized AMP that may normally function in the innate immune system. This finding stands in stark contrast to current models of Aβ-mediated pathology and has important implications for ongoing and future AD treatment strategies".

Aβ AS AMP AGAINST INFECTIOUS VIRUSES

Aβ-dependent inhibition of influenza virus replication

A new picture is slowly emerging with respect to a protective role of Aβ against viral infection. Three recent publications have reported this property in inhibition of replication of influenza [177] and HSV-1 virus [178, 179]. For instance, White et al. [177] have investigated the antiviral effect of $A\beta_{40}$ and $A\beta_{42}$ peptides on the replication of seasonal H3N2 and pandemic H1N1 strains of influenza A virus *in vitro*. Influenza viruses are enveloped RNA viruses that belong to the *Orthomyxoviridae* family [180, 181]. They are highly contagious and cause acute respiratory distress. Influenza viruses are still the cause of significant morbidity and mortality worldwide. The most severe recorded pandemic occurred in 1918 and has been known as the *Spanish flu* which caused 40 millions deaths [182]. According to their core protein, influenza viruses are classified into three types, A, B, and C [180, 183]. The influenza A viral particle possesses a lipid envelope, which is derived from the host's cell membrane during the process of virus budding and three virus-specific envelope-embedded proteins which are haemagglutinin, neuraminidase, and matrix ion channels (Fig. 3). The virus binds to sialic acid-galactose decorated glycoprotein receptors on the surface of respiratory epithelial cells, fuses with the host plasma membrane, is internalized and transfers its genetic material to the nucleus [184, 185].

Table 2
AMP activity of Aβ peptides, control and LL-37

Minimal inhibitory concentrations (μg/ml)						
Organism	$A\beta_{42}$	$A\beta_{40}$	$roA\beta_{42}$	LL37	$reA\beta_{42}$	$scA\beta_{42}$
Candida albicans	0.78	0.78	0.78	6.25	>25	>50
Escherichia coli	1.56	1.56	3.13	1.56	>50	>50
Staphylococcus epidermidis	3.13	50	3.13	25	>50	>50
Streptococcus pneumoniae	6.25	12.5	6.25	1.56	50	>50
Staphyloccus aureus	6.25	25	12.5	6.25	>50	>50
Listeria monocytogenes	6.25	25	6.25	25	>50	50
Enterococcus faecalis	6.25	50	3.13	6.25	50	>50
Streptococcus agalactiae	12.5	50	>50	12.5	>50	>50
Pseudomonas aeruginosa	>50	>50	>50	6.25	>50	>50
Streptococcus pyogenes	>50	>50	>50	6.25	>50	>50
Streptococcus mitis	>50	50	>50	6.25	>50	>50
Streptococcus salivarius	>50	>50	>50	50	>50	>50

The antimicrobial activity of synthetic Aβ1-42 ($A\beta_{42}$), Aβ1-40 ($A\beta_{40}$), rodent Aβ1-42 ($roA\beta_{42}$), LL-37 (LL-37), reverse Aβ42-1 ($reA\beta_{42}$), or scrambled Aβ42 ($scA\beta_{42}$) peptides were determined as minimal inhibitory concentrations (MIC) against 12 microorganisms. Reproduced from [174].

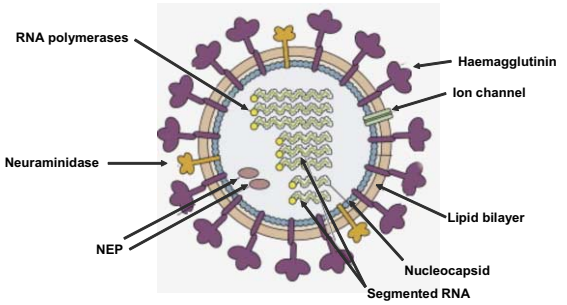

Fig. 3. Schematic representation of influenza virus.

In the case of White et al.'s work [177], the authors showed that Aβ peptides displayed neutralizing activity when either strain of virus was preincubated with the Aβ peptides in assays of infection of two different epithelial cell lines. Of interest, data showed that the $A\beta_{42}$ isoform had greater activity than the $A\beta_{40}$ isoform. Of significance, data suggested that Aβ peptides established interactive bonds with the virus. This interpretation was further confirmed by turbidimetry assays (Fig. 4) and confocal experiments that showed that Aβ induced aggregation of influenza virus. In addition, it was shown that Aβ peptides reduced viral uptake by epithelial cells, increased virus uptake by neutrophils and reduced pro-inflammatory cytokine IL-6 production by these cells. The authors did not provide a definitive mechanism of action of Aβ in these studies but suggested the possibility that Aβ-dependent interference of influenza virus infectivity could be related to alteration of the integrity of the viral envelope,

AMP activity of Aβ peptides against HSV-1 replication in vitro

HSV-1 is a member of the *Herpesviridae* family of virus that causes lifelong latent infections. It is a double-stranded DNA virus composed of a linear genome. From a structural standpoint, it is composed of an external envelope derived from the nuclear membrane of the host cell that is acquired in the process of virus budding. The envelope is made of a lipid bilayer membrane and decorated with several types of membrane-embedded glycoproteins that protect the encapsidated DNA and its tegument proteins [186] (Fig. 5).

The first step in HSV-1 infection is attachment and fusion of the viral envelope with the cell plasma membrane. Three glycoproteins of the viral envelope play a central role in attachment and infection of target cells [187, 188]. These glycoproteins are

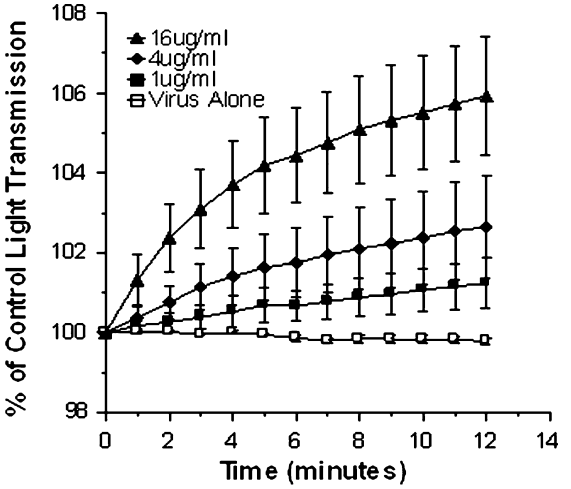

Fig. 4. Viral aggregation induced by $A\beta_{42}$. Aggregation of the Aichi68 H3N2 viral strain as assessed by measurements of increased light absorbance at 350 nM through a stirred viral suspension. Aggregation was significant ($n = 5$, $p < 0.02$) at the 16 μg/ml concentration of $A\beta_{42}$ as compared to light transmission through the control virus preparation. (Reproduced from [177], White et al. (2014) *PLoS One* 9, e101364.).

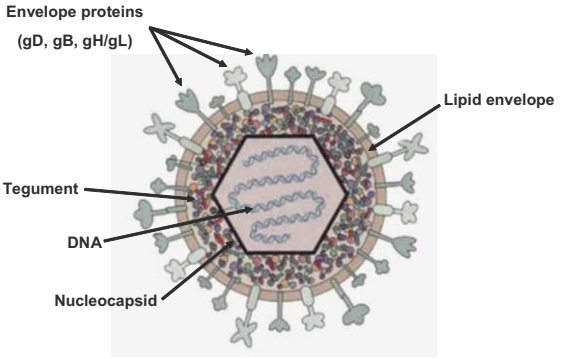

Fig. 5. Schematic representation of HSV-1 virus.

glycoprotein B (gB) and heterodimeric glycoprotein H/glycoprotein L (gH/gL). A number of additional viral envelope proteins participate in cognate cellular receptor recognition to ensure viral tropism [189]. This process induces formation of pores, which allow entry of the DNA-containing nucleocapsid into the cytoplasm [189]. gB is a single-pass glycoprotein that comprises 904 amino acid residues that are organized into five regions/domains according to the tridimensional structure of the protein [190]. These are the ectodomain (positions 1–774), the membrane proximal region (MPR), which extends from positions 713 to 763, the transmembrane domain (positions 775–795) and the cytoplasmic domain (positions 796–904).

Bourgade et al. [178, 179] have reported results of investigations designed to answer the question whether $A\beta_{40}$ and $A\beta_{42}$ peptides would behave as AMP in *in vitro* assays of HSV-1 infection of fibroblast, epithelial and neuroglioma cell lines. Data showed that both Aβ isoforms but not scrambled peptides (control), inhibited HSV-1 replication in these cells when added 2 h prior to or concomitantly with virus challenge (Fig. 6). Of significance, Aβ peptides were inefficient when added 2 or 6 h after exposing the cells to the virus. In contrast, comparative experiments using LL-37, a recognized **bona fide** AMP [176], revealed that the mode of action of Aβ peptides differed dramatically. Of significance, the inhibitory effect of Aβ was not observed when cells were challenged with an adenovirus (hAd5). These observations suggested that 1) Aβ peptides bound to HSV-1, as in the case of Aβ interaction with influenza virus [177], 2) the result of these interactions created interference with the process of viral fusion with the target cells, 3) Aβ interacted with HSV-1, a virus that possesses an envelope but not with envelope-free adenovirus, 4) the AMP effect of Aβ differed from that of LL-37 and, 5) Aβ initiated their anti-viral effect prior to HSV-1 entry into the cells. The bulk of these observations led the authors to conclude that, "Aβ peptides represent a novel class of antimicrobial peptides that protect against neurotropic enveloped virus infections such as HSV-1. Overproduction of Aβ peptide to protect against latent herpes viruses and eventually against other infections, may contribute to amyloid plaque formation, and partially explain why brain infections play a pathogenic role in the progression of the sporadic form of AD."

The same group sought [179] to obtain additional evidence regarding the protective role of Aβ against HSV-1 infection in *in vitro* co-cultures of neuroglioma (H4) and glioblastoma (U118-MG) cell lines, a model that allows the study of the mutual influence of these cells in the production/effect of Aβ. Whereas H4 cells produced appreciable levels of HSV-1 upon initial infection, the level of virus production did not increase with continued incubation. Addition of a BACE-1 inhibitor to prevent Aβ production increased HSV-1 production several fold, suggesting an inhibitory effect due to endogenous Aβ generation. Quantification of $A\beta_{42}$ in HSV-1-infected H4 cells confirmed a robust production of $A\beta_{42}$ in the supernatant in response to HSV-1 infection. As expected, U118-MG cells produced low

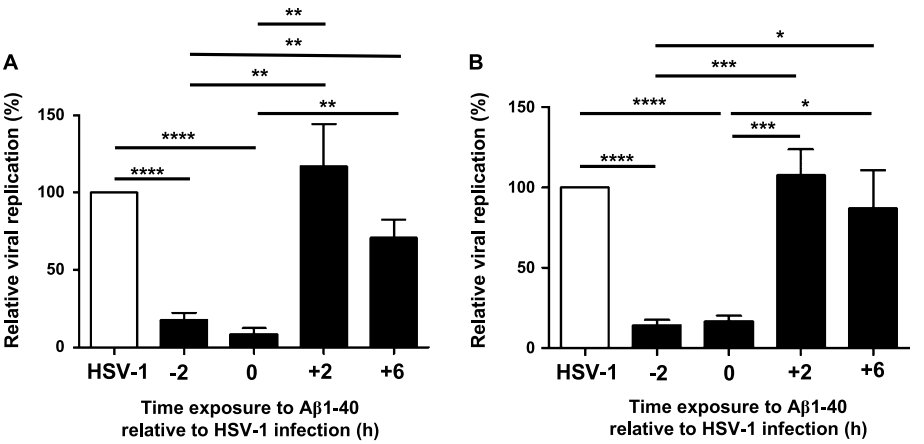

Fig. 6. $A\beta_{40}$ and $A\beta_{42}$ inhibition of HSV-1 replication in MRC-5 cells. MRC-5 cells were exposed to HSV-1 (0.01 ID50 per cell) and, $A\beta_{40}$ (20 μg/ml) (A) or $A\beta_{42}$ (20 μg/ml) (B) were added 2 h before, simultaneously or 2 and 6 h after exposing the cells to the virus. Viral replication was stopped after 24 h by freezing the cell suspension. DNA was isolated and aliquots were analyzed by real-time PCR. Results were computed as the ratio of viral DNA relative to β-actin DNA. Data are shown relative to HSV-1 replication in the absence of Aβ peptides arbitrarily set at 100%. They are displayed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (Reproduced from [178], Bourgade et al. (2015) *Biogerontology* **16**, 85-98, with permission from Springer provided by the Copyright Clearance Center).

levels of $A\beta_{42}$. However, $A\beta_{42}$ production in co-cultures was low, suggesting interference due to the presence of glioblastoma cells or uptake of $A\beta_{42}$ by these cells. Confocal experiments confirmed that glioblastoma cells captured $A\beta_{42}$, in agreement with what has been reported for microglial cells in Aβ clearance in the brain [40, 132]. Further evidence for the protective effect of $A\beta_{42}$ against HSV-1 replication was obtained by transfer of supernatants of H4 cells infected with HSV-1 (conditioned supernatants) that were assayed in *de novo* cultures of H4 cells challenged with HSV-1. It was found that conditioned supernatants conferred protection against HSV-1 infection, likely due to the presence of Aβ because similar supernatants generated by BACE-1 inhibitor-treated H4 cells were ineffective. The protection was not due to the presence of interferon alpha in the supernatants (Fig. 7). The authors concluded, "Our data reinforce the recent thinking that Aβ may be beneficial and not simply a byproduct in the pathogenesis of AD. It may be suggested that therapeutic agents should target the aggressors that induce Aβ production such as HSV-1 infection rather than Aβ because of its frequent detection in the brains of AD patients".

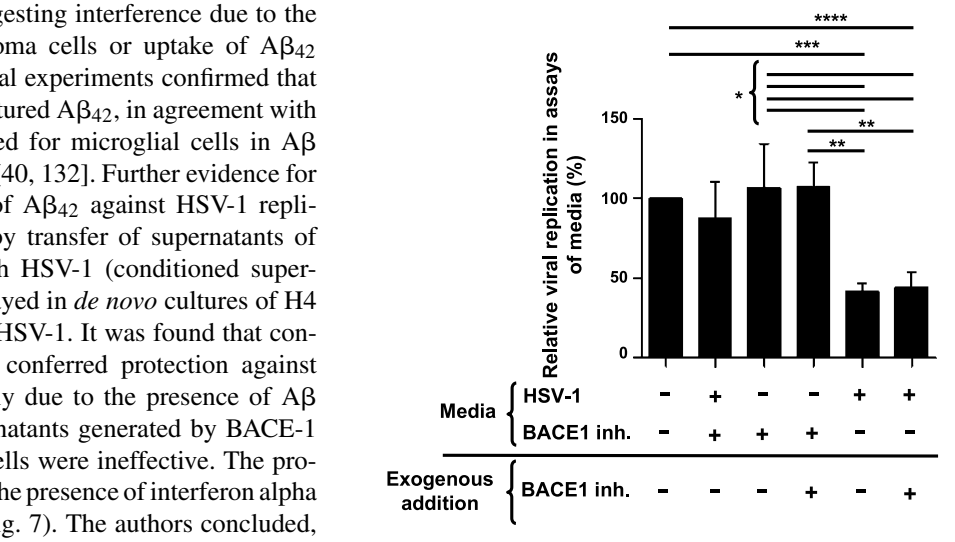

Fig. 7. Assays of media of H4 cells cultured under various conditions on HSV-1 replication in *de novo* cultures of H4 cells. A) Media were obtained from 24 h cultures of H4 cells grown in the presence/absence of HSV-1 or, in the simultaneous presence/absence of BACE-1 inhibitor (BACE1 inh.), as indicated. Exogenous BACE-1 inhibitor was added to some samples prior to assays, as indicated (Exogenous addition). Media were individually assayed by addition to *de novo* cultures of H4 cells 2 h before challenge with HSV-1 for 24 h. Viral replication was quantitated by real-time PCR. Data are displayed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Reprinted from [179], *Journal of Alzheimer's Disease*, Volume 50, Karine Bourgade, Aurélie Le Page, Christian Bocti, Jacek M. Witkowski, Gilles Dupuis, Eric H. Frost and Tamàs Fülöp Jr., Protective effect of amyloid-β peptides against herpes simplex virus-1 infection in a neuronal cell culture model, pages 1227-1241, Copyright (2016), with permission from IOS Press.

PUTATIVE MECHANISM OF Aβ-DEPENDENT INHIBITION OF HSV-1 INFECTION

There has not been any clear molecular mechanism suggested to explain the AMP activity of Aβ

in inhibition of infection by HSV-1. However, Bourgade et al. have suggested that sequence homology between the MPR of the gB fusion protein [191] and Aβ could provide a clue in this matter [178]. In this connection, it has been shown that the MPR serves to temporarily cover or shield lipid-associating moieties or fusion loops of gB [192]. The homology of sequence of Aβ peptides suggests that they could bind to these fusion loops and prevent the fusion process. Overall, the combined results [178, 179] of Bourgade et al. led to the following observations:

- Aβ did not enter the cells that are the target of HSV-1, as shown by confocal experiments.
- The anti-viral action of Aβ action occurred outside of the target cell.
- Aβ loosely interacted with target cells, as shown by washing experiments.
- Aβ protection was related to the time-sequence of its addition to cells challenged with HSV-1.
- Aβ anti-viral protection was efficient before or concomitant with HSV-1 challenge but not after HSV-1 had time to fuse with target cells.
- HSV-1 did not enter Aβ-treated cells.
- Aβ protective effect was observed in the case of an enveloped virus but not an envelope-free virus.
- Aβ bound to HSV-1, as shown by experiments in a cell-free system and molecular proximity FRET experiments (unpublished).
- Sequence homology between the MPR region of gB and Aβ suggested that this region of the fusion protein which is important to maintain protein stability and viral fusion [192] could be an intra target site resulting in interference with viral fusion.

Can the MPR region of fusogenic HSV-1 gB be a target of Aβ?

gB is a conserved protein essential to the cell-entry machinery of herpes viruses. Its MPR region is very hydrophobic and thought to lie in juxtaposition to the plasma membrane, facilitating merger of the HSV-1 envelope. It is also thought to form a pedestal for the trimeric ectodomain of gB [193] and to shield the fusion loops prior to gB triggering of viral fusion [192, 194]. Herpes infection is a multi-stage process that initially involves viral glycoproteins gD, gB, gH, and gL and their binding to the cellular membrane components nectin-1, herpesvirus entry mediator and a modified heparan sulfate [187, 195]. gB MPR region plays a key role in herpes virus association with the target cell and viral fusion and entry. For instance, mutations of non-variant residues in the MPR region markedly decreased infectivity of HSV-1 in *in vitro* assays [196]. In addition, Hannah et al. [197] have performed a series of mutations (deletion, truncation) in the MPR region and these have revealed that the purified mutant proteins failed to bind to liposomes. These observations led the authors to conclude, "that the ability of the herpes simplex virus (HSV) glycoprotein B (gB) fusion protein to interact with the host membrane is regulated by its membrane-proximal region (MPR), which serves to cover or shield its lipid associating moieties (fusion loops). This in turn prevents the premature binding of gB with host cells and provides a level of regulation to the fusion process". The bulk of these observations, along with those that presented evidence for a relationship between MPR and the ability of the fusion loops of gB to associate with the plasma membrane [194, 197], provide solid arguments for a regulatory role of MPR in the initial steps of HSV-1 infection. The question thus arises to explain the antiviral effect of Aβ against HSV-1 infectivity. An intriguing possibility to account for the selective effect of Aβ peptides may reside in the fact of the homology of sequence between the MPR and Aβ, as depicted (Fig. 8). Aβ could compete with the MPR region and (partially) disturb the pre-fusion structure of gB, thus altering the spatial arrangement of its fusion loops and inhibiting its action which is essential for HSV-1 fusion [197]. If this hypothesis were valid, it would support the series of observations of Bourgade et al. [178, 179] (outlined above) and provide a working framework for further investigations concerning the mechanism of the antiviral AMP properties of Aβ peptides toward (enveloped) HSV-1. In addition, the mechanism may provide novel avenues in the management of AD. However, Bourgade et al.'s data [178, 179] did not exclude the possibility of a selective alteration of the HSV-1 envelope as a result of pore formation by Aβ that would result in inhibition of HSV-1 infection.

FUTURE DIRECTIONS

The findings that Aβ display antimicrobial [174] and antiviral [177–179] activity open the way to a novel concept concerning the physiological role of these peptides as protectors against pathogenic

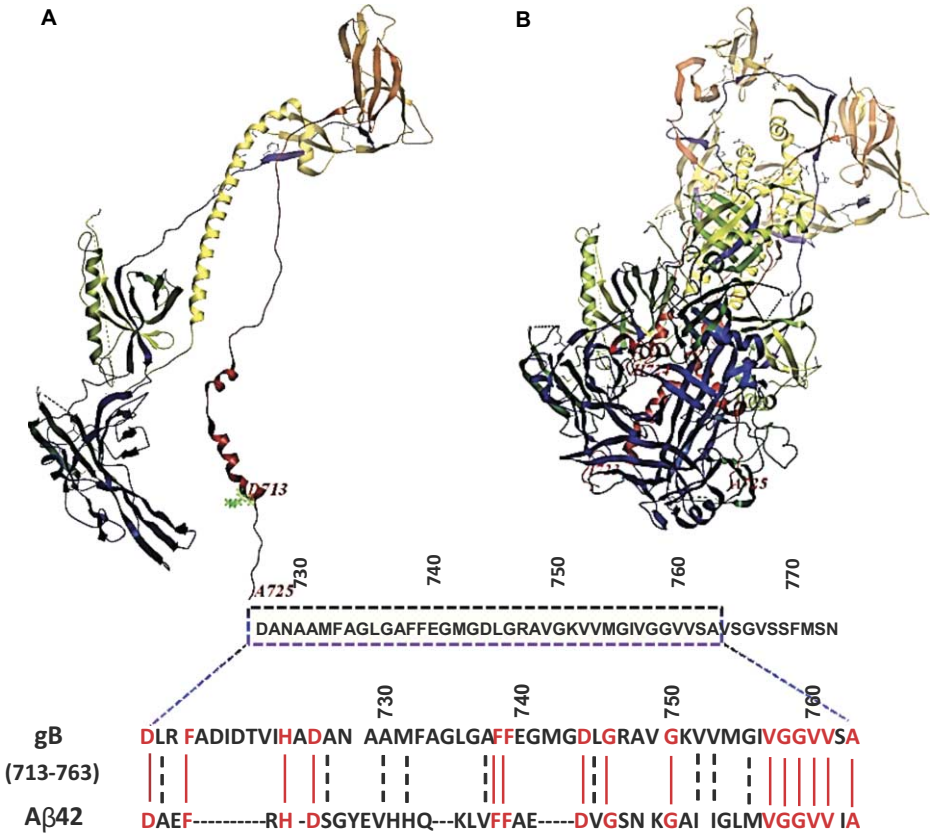

Fig. 8. Amino acid sequence homology between the membrane proximal region (MPR) of HSV-1 fusogenic glycoprotein gB and $A\beta_{42}$. A) Amino acid sequence alignment of $A\beta_{42}$ and MPR (positions 713–773) of HSV-1 gB using the Clustal Omega shareware (http://expasy.org/proteomics). The gB sequence shown corresponds to HSV-1 (strain F) that is archived in UniProtKB/Swiss-Prot (accession number, P06436) SIB Bioinformatics Resource Portal (http://expasy.org/). Identical amino acid residues are indicated by vertical red lines and amino acids possessing similar properties, by dashed vertical black lines. Adapted with permission from [191], Cribbs et al. (2000) *Biochemistry*, **39**, 5988-5994, Copyright 2000, American Chemical Society. The ectodomain of the three dimensional structure of one monomer (monomer a) of trimeric gB glycoprotein determined by X-ray crystallography [190], is illustrated, with the partial C-terminal section of the protein sequence shown in one letter amino acid code, and numbered. B) Three-dimensional structure of native gB [190] showing the trimeric form. (RCSB Protein Data Bank, http://www.rcsb.org, PDB ID 2GUM).

aggression of the brain rather than being exclusively associated with cytotoxic components of AD. From a mechanistic standpoint, data are consistent with Aβ interaction with viruses [177, 178]. We propose a minimal model whereby Aβ peptides do not enter the target cells but interfere with HSV-1 infection prior to its fusion with the cell plasma membrane (Fig. 9), thereby inhibiting infection by HSV-1.

However, many questions remain unanswered concerning the AMP property of Aβ peptide:

- *From the standpoint of the physiological properties of Aβ*
  - Can the observations made in the case of Aβ-dependent interference of HSV-1 infectivity be extended to other members of the Herpesviridae family and to viruses of other families?
  - Could an analogous induction-for-protection model be evoked for bacterial and, notably spirochete latent infection that would lead to overproduction of Aβ?
  - Are Aβ peptides involved in this protective process in the brain?
  - Could vaccination against *Herpesviridae* viruses early in life protect against development of LOAD, based on the observation that a large proportion of AD patients are infected with HSV [48]?
- *From the standpoint of future basic research concerning the mechanism of action of Aβ*

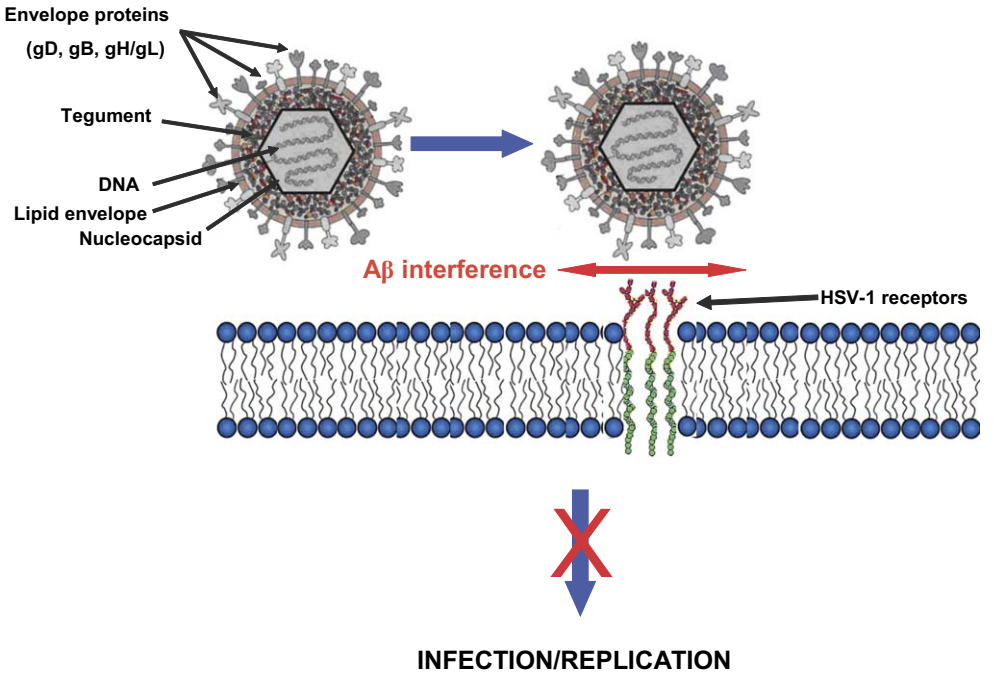

Fig. 9. Minimal model illustrating the mode of action of Aβ in preventing HSV-1 infection. HSV-1 attaches to the plasma membrane of the target cell by way of interaction of its fusogenic g;ycoproteins with plasma membrane receptors. Aβ interacts with HSV-1, presumably through fusogenic gB glycoprotein, and interferes with its binding/entry into the cell.

- What is the minimal Aβ sequence required to inhibit HSV-1 infectivity of target cells in functional assays?
- What are the critical amino acid residues of the Aβ sequence essential for their AMP property? Analogs of biological interest could be further analyzed for their ability to assemble and to form fibrils [198] using biophysical techniques [199–201].
- Would the MPR sequence inhibit HSV-1 infection?
- Does Aβ (or analogs) interference occur in lipid rafts, which are the preferred site of HSV-1 entry into target cells [202]?
- Does the binding of Aβ (or analogs) to gB induce conformational changes? This possibility could be addressed using a GFP-labelled gB that can be functionally expressed in infectious HSV-1 [203].

CONCLUSIONS

Accumulation of Aβ results from insults to the brain and this is a contributing factor to the development of AD. Unraveling the mechanism leading to Aβ accumulation and deposit is of crucial importance to design ways and treatments of this devastating disease. Furthermore, the discovery that Aβ may confer an early protective role as AMP to fight various microbial aggressions in the brain, including HSV-1, opens additional avenues in understanding the complex picture of AD. On the one hand, the role of Aβ as AMP has to be taken into account as an essential component of protection of the brain and, therefore, must be reckoned with in the design of medical interventions that are aimed at eliminating Aβ. On the other hand, the harmful effects of Aβ as cytotoxic products of AβPP processing generated under conditions of brain aggression remain a focus point in the design of targeted prevention/treatment of AD. Understanding the mechanisms that lead to the fine regulation of AβPP processing through the non-amyloidogenic and amyloidogenic pathways, as well as the products of AβPP processing, is a timely and pressing challenge that needs to be resolved to tackle the ongoing threat of AD on a worldwide basis. Time is of the essence and this human problem ought to be among the highest priorities of governing authorities.

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NOTE ADDED IN PROOFS

A provocative paper by Kumar et al. [204] has recently extended the original findings of Soscia et al. [174]. This publication provides solid evidence for the role of Aβ as AMP against fungal *(Candida albicans)* and bacterial (*Salmonella enterotica* serotype *typhimurium)* infections in cultured human cell lines and, in transgenic Aβ-expressing nematodes *(Caenorhabditis elegans)* and mice. The authors used cultures of human brain neuroglioma (H4) cells and chinese hamster ovary (CHO) cells to assess resistance to *C. albicans.* Results showed that survival of $A\beta_{40}$- and $A\beta_{42}$-expressing H4 and CHO cells was significantly increased as compared to wild-type cells. Of significance, it was observed that supernatants of Aβ-expressing cell cultures were able to form fibrils and oligomers that entangled and clumped *C. albicans.* These observations gave a clue with respect to the mode of action of Aβ peptides as bona fide AMP. In addition, *in vivo* experiments using C. elegans engineered to express human $A\beta_{42}$ revealed that these transgenic worms survived three to four more days following infection of the gut with *C. albicans* or *S. typhimurium,* compared to wild-type worms that did not express $A\beta_{42}$. Four-week-old transgenic (5XFAD) mice that constitutively expressed human Aβ at high levels in the brain but that do not show deposits of Aβ and features of neuroinflammation, were infected intracerebally with *S. typhimurium.* Control animals were non-transgenic wild-type littermates. Results showed rapid seeding and acceleration of Aβ deposits in the brain of 5XFAD mice that colocalized with invading bacteria which became entangled within fibrils of Aβ deposits. Control mice did not show these features. Of significance, survival of transgenic mice was significantly increased with respect to controls. However, both groups of mice succumbed to infection, suggesting that expression of Aβ conferred only partial resistance to bacterial infection in the brain.

The bulk of the data reported in Kumar et al.'s publication led the authors to suggest a model in which soluble Aβ oligomers initially bind to a heparin domain(s) of the microbial cell wall carbohydrates. Propagating Aβ fibrils initially mediate pathogen agglutination, followed by entrapment of the invading microbes. Aβ recognition of the heparin domain likely involves the peptidic sequence XBBXBX (where X is a hydrophobic or uncharged amino acid residue and B is a basic amino acid residue) that is present in Aβ (positions 12–17, VHHQKL) and in human cathelicidin LL37. The authors concluded that, "Our data are consistent with a protective role for Aβ in innate immunity that uses a classic AMP mechanism characterized by reduced microbial adhesion to host cells and agglutination and entrapement of microbes by Aβ fibrils". Importantly, Kumar et al.'s data lend further support to the notion that Aβ may play a protective role in innate immunity as an additional line of defense against infectious or sterile inflammatory stimuli.

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Herpes Simplex Virus Type 1 and Other Pathogens are Key Causative Factors in Sporadic Alzheimer's Disease

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Abstract. This review focuses on research in epidemiology, neuropathology, molecular biology, and genetics regarding the hypothesis that pathogens interact with susceptibility genes and are causative in sporadic Alzheimer's disease (AD). Sporadic AD is a complex multifactorial neurodegenerative disease with evidence indicating coexisting multi-pathogen and inflammatory etiologies. There are significant associations between AD and various pathogens, including Herpes simplex virus type 1 (HSV-1), Cytomegalovirus, and other *Herpesviridae*, *Chlamydophila pneumoniae,* spirochetes, *Helicobacter pylori,* and various periodontal pathogens. These pathogens are able to evade destruction by the host immune system, leading to persistent infection. Bacterial and viral DNA and RNA and bacterial ligands increase the expression of pro-inflammatory molecules and activate the innate and adaptive immune systems. Evidence demonstrates that pathogens directly and indirectly induce AD pathology, including amyloid-β (Aβ) accumulation, phosphorylation of tau protein, neuronal injury, and apoptosis. Chronic brain infection with HSV-1, *Chlamydophila pneumoniae*, and spirochetes results in complex processes that interact to cause a vicious cycle of uncontrolled neuroinflammation and neurodegeneration. Infections such as Cytomegalovirus, *Helicobacter pylori*, and periodontal pathogens induce production of systemic pro-inflammatory cytokines that may cross the blood-brain barrier to promote neurodegeneration. Pathogen-induced inflammation and central nervous system accumulation of Aβ damages the blood-brain barrier, which contributes to the pathophysiology of AD. Apolipoprotein E4 (ApoE4) enhances brain infiltration by pathogens including HSV-1 and *Chlamydophila pneumoniae*. ApoE4 is also associated with an increased pro-inflammatory response by the immune system. Potential antimicrobial treatments for AD are discussed, including the rationale for antiviral and antibiotic clinical trials.

Keywords: Alzheimer's disease, ApoE4, amyloid, Cytomegalovirus, dementia, Herpes simplex, neurodegeneration, pathogen

THE ALZHEIMER'S DISEASE PATHOGEN HYPOTHESIS

Alzheimer's disease (AD) is an inflammatory brain disease that affects 20 million people worldwide and the incidence is expected to rise. Current medical treatment is not optimal, and thus an effective treatment is very much needed. The disease is associated with a combination of environmental agents and genetic influences leading to inflammation of the brain, neuronal cell death, and progressive dementia [1].

AD is characterized by two main pathological features in the brain: senile plaques and neurofibrillary tangles (NFTs). Senile plaques are extracellular and are predominantly made up of amyloid-β (Aβ), a peptide cleaved from the much longer amyloid-β protein precursor (AβPP). Neurofibrillary tangles are intracellular and comprised of abnormally phosphorylated tau protein. Tau protein is normally associated with microtubules in neurons, and contributes to AD pathology in its phosphorylated state [2].

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The AD pathogen hypothesis states that pathogens act as triggers, interacting with genetic factors to initiate the accumulation and/or formation of Aβ, hyperphosphorylated tau proteins, and inflammation in the AD brain. Herpes simplex virus type 1 (HSV-1) and other pathogens including *Chlamydophila pneumoniae* and Spirochetes are able to infect the brain, evade the host immune response, and are highly prevalent in the AD brain [3–9]. *In vitro* studies and animal models indicate that pathogens induce formation of Aβ, amyloid plaques, and hyperphosphorylated tau proteins [10–13]. Pathogens induce a glial inflammatory response and can directly and indirectly damage and destroy neurons [14–18]. Significant inflammatory cascades are activated in the brains of AD patients [19, 20]. Together, these processes result in neurodegeneration and disease progression.

This review examines evidence implicating HSV-1 and Cytomegalovirus (CMV), both members of the *Herpesviridae* family, and the bacterial pathogens *Chlamydia pneumoniae*, spirochetes, periodontal pathogens, and *Helicobacter pylori* as causative in the pathogenesis of AD. Limited evidence is also presented regarding the *Herpesviridae* Epstein Barr Virus (EBV) and Human herpes virus 6 (HHV-6) as possible contributing factors in AD pathogenesis. The multi-pathogen AD hypothesis does not exclude toxins or other environmental co-factors that may be involved in the pathogenesis of AD and are reviewed elsewhere [21]. Pathogens were selected based on the degree of significant cumulative evidence identified in an extensive PubMed literature search.

HERPES SIMPLEX VIRUS TYPE 1

HSV-1 is a neurotropic virus that infects most humans, attaining 90% prevalence by the sixth decade of life. Infection is life long, as the virus resides in the trigeminal ganglia of the peripheral nervous system in latent form with viral genome but no virions present. Reactivation leads to viral replication and acute infections known as herpes labialis, commonly referred to as cold sores [22].

In 1982, Melvin Ball hypothesized that HSV-1 was causative in AD. He proposed that latent HSV-1 located in the trigeminal ganglia could reactivate and ascend along known nerve pathways into the limbic system and areas of the brain most affected in AD [23].

Herpes simplex encephalitis and AD affect the same brain regions, including the frontal lobes, temporal lobes, and hippocampus. Herpes simplex encephalitis survivors show cognitive, memory, and behavioral decline. Other viruses implicated in neurological disease include measles in subacute sclerosing panencephalitis and human immunodeficiency virus in HIV-associated dementia [22]. As with AD, both subacute sclerosing panencephalitis [24] and HIV infection [25] are associated with the formation of phosphorylated tau protein and NFTs in the brain.

EPIDEMIOLOGICAL STUDIES: HSV-1 HUMORAL RESPONSE, COGNITIVE DECLINE, AND AD

Epidemiological studies show an association between viral infectious burden (IB) and cognitive decline. IB is defined as a composite serological measure of exposure to common pathogens [27]. Strandberg *et al*. measured seropositivity to HSV-1, HSV-2, CMV, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae* in 383 elderly patients with cardiovascular disease. Assessments including the Mini-Mental Status Examination (MMSE) and the Clinical Dementia Rating were used to define cognitive impairment. Having three positive viral titers was associated with a 2.5 times higher risk for cognitive impairment after 12 months [26]. Katan *et al*. [27] found an association between *Herpesviridae* and cognitive decline using a composite serologic measure of exposure to both bacterial (*Chlamydia pneumoniae* and *Helicobacter pylori*) and viral (CMV, HSV-1, and HSV-2) pathogens. As reviewed by Strandberg, the association was primarily driven by viral IB [28].

Letenneur *et al*. studied the risk of developing AD according to the presence or absence of serum anti-HSV IgG and IgM antibodies by following 512 elderly patients initially free of dementia for 14 years. The presence of anti-HSV IgM antibodies is associated with primary infection or recent reactivation of HSV. In contrast, the presence of anti-HSV IgG antibodies indicates lifelong HSV infection [29]. Subjects who were IgM-positive at baseline showed a significantly higher risk of developing AD (hazard ratio = 2.55). No significant increased risk for AD was found in IgG-positive subjects. Among the 43 IgM-positive subjects, only 2 were IgG-negative, which supports recent HSV reactivation rather than primary infection in most of the IgM-positive subjects [29].

Similar results were obtained in a longitudinal study by Lövheim *et al*. involving 3,432 elderly patients with a mean follow-up time of 11.3 years. Baseline increased serum levels of anti-HSV IgM antibodies were associated with increased risk of developing AD by a factor of 2 [30]. Thus, HSV reactivation, as indicated by the presence of anti-HSV IgM antibodies, is highly correlated with incident AD [29, 30].

Kobayashi *et al*. [31] used the avidity index of anti-HSV-1 IgG antibodies as an indicator of HSV-1 reactivation. The study, involving patients with amnestic mild cognitive impairment (MCI), AD, and healthy controls evaluated the relationship between HSV-1 reactivation and the degree of cognitive impairment in AD. The avidity index is defined as the strength with which IgG attaches to antigen [32]. HSV reactivation is characterized by increased levels of high-avidity anti-HSV IgG antibodies compared to lower levels seen with initial HSV infections [31]. MMSE and frontal assessment battery were used to assess cognition. MCI patients had a higher anti-HSV-1 IgG antibody avidity index than AD patients or healthy controls implying that HSV-1 reactivation occurs more frequently in the MCI group than in the AD group or healthy control group. Differences in anti-HSV-1 IgG antibody titer and anti-HSV-1 avidity index readings between the MCI group and healthy controls also suggests that reactivation of HSV-1 contributes to progression from healthy state to MCI [31].

In a longitudinal nested case–control study, Lövheim *et al*. measured plasma HSV antibody samples taken on average 9.6 years before AD diagnosis. In the 360 patients who developed AD and who had a follow-up time of 6.6 years or more, past HSV infection (as indicated by the baseline presence of anti-HSV IgG antibodies) increased the risk of developing AD by a factor of 2.25 [33].

Schretlen *et al*. evaluated cognitive performance in a group of patients who had been diagnosed with schizophrenia with an average cohort age of 39 years [34]. Schizophrenia patients who were HSV-1 IgG antibody seropositive performed significantly worse on neuropsychological measures (including psychomotor speed, executive functioning, and explicit verbal memory) than the combined HSV-1 and HSV-2 IgG antibody seronegative control group. Patients who tested seropositive for HSV-1 had decreased grey matter volume in the anterior cingulate and cerebellum seen on morphometric magnetic resonance imaging (MRI) of the brain compared to the HSV-1 seronegative control group [34]. Poor cognitive test performance correlated with decreased grey matter volume in some of the same brain regions that distinguished the patient subgroups defined by HSV-1 status [34]. Several studies have confirmed significant cognitive impairment in HSV-1 IgG seropositive schizophrenia patients compared to HSV-1 IgG seronegative controls with average cohort ages reported as 38 to 42 years old [35–39]. A causal association between exposure to HSV-1 and increased risk for schizophrenia has not been proven [34]; however, HSV-1 exposure in this group of neuropsychiatric patients is associated with cognitive impairment and provides further supportive evidence for the role of HSV-1 in cognitive dysfunction.

Higher levels of HSV-1 humoral immune response appear to play a protective role in the early stages of AD. Analyses performed with voxel-based morphometric brain MRI in AD patients and healthy controls indicate the presence of significant correlations between the preservation of cortical bilateral temporal and orbitofrontal grey matter volumes with higher HSV-1 IgG serum antibody titers [40].

ADDITIONAL STUDIES ASSOCIATING EXPOSURE TO HSV-1 AND OTHER *HERPESVIRIDAE* WITH COGNITVE IMPAIRMENT AND AD

In a recent historical prospective study, Fruchter *et al*. [275] evaluated 612 young healthy soldiers by measuring the effect of HSV-1 IgG seropositivity on cognitive function and language abilities. The average age of subjects was 17 years at study initiation. Subjects exposed to HSV-1 infection had significantly lower language skills and IQ scores compared to seronegative subjects after controlling for sex, immigration status, and education. The findings remained significant after removal of individuals diagnosed with mental illnesses at the time of recruitment. Fruchter *et al*. present one of the first studies to show the detrimental effect of HSV-1 exposure on cognition and language in young, healthy, non-psychiatric patients [275]. These findings corroborate a study by Tarter *et al*., which revealed an association between HSV-1 seropositivity and impaired cognition across all age groups, including children and middle-aged adults [87].

Gale *et al*. evaluated the relationship between infectious burden and cognitive function in 5,662 young to middle-aged subjects from 20 to 59 years-of-age [276]. The infectious burden index consisted of an aggregate measure of exposure to HSV-1,

HSV-2, CMV, hepatitis A, hepatitis B, hepatitis C, toxoplasmosis, and toxocariasis, as determined by IgG antibodies to these pathogens. Controlling for age, sex, education level, race-ethnicity, and poverty-to-income ratio, infectious burden index was associated with a significant decrease in cognitive function, as measured by symbol-digit substitution and serial-digit learning. HSV-1, CMV, and hepatitis A had higher prevalence and higher coefficients associating infectious burden with decreased cognitive outcomes than the other pathogens in the study, suggesting that these three pathogens largely contributed to the findings [276].

Steel *et al*. performed a meta-analysis involving 35 studies which identified viral DNA from various *Herpesviridae* in brain or peripheral blood leukocytes (PBLs), and/or antibody seropositivity in patients who had been diagnosed with AD [277]. The meta-analysis included studies involving HSV-1, HSV-2, CMV, EBV, HHV6, and Varicella zoster virus (VZV). Collective analysis revealed that the presence of *Herpesviridae* in the brain was associated with an increased risk of AD compared to controls (OR 1.38; 95% CI 1.14–1.66). Subgroup analysis showed that infection with HSV-1 (OR 1.38), HHV6 (OR 2.23), CMV (OR 1.20), or EBV (OR 1.55) was associated with an increased risk of AD compared to controls. Possession of the *APOE-ε*4 allele together with HSV-1 positivity increased the risk of developing AD (OR 2.25) [277].

HSV-1 IS HIGHLY PREVALENT IN ELDERLY BRAINS

Polymerase chain reaction (PCR) methods used by Jamieson *et al*. to detect HSV-1 DNA in autopsy brain specimens confirmed that latent HSV-1 is present in a high proportion (70–100%) of sporadic AD and elderly normal brains [4]. HSV-1 was found in brain areas most affected by AD, namely the temporal cortices, frontal cortices, and hippocampus. The Jamieson *et al*. findings have been confirmed in several studies (Table 1) [41]. The virus was found in very low proportions in younger brains [42]. In addition, Mori *et al*. [43] and Rodriguez *et al*. [44] used PCR to identify HSV-1 DNA in AD brains. PCR improves sensitivity in HSV-1 detection when compared to previously applied techniques such as *in situ* hybridization [4]. Some PCR studies had lower detection rates than others, perhaps due to a lower prevalence of HSV-1 infection in Japan [45] or age not having been taken into account. For unknown reasons, Hemling *et al*. [46] and Marquis *et al*. [47] detected HSV-1 DNA in a very low proportion of brains.

Intrathecal HSV-1 IgG was found in 52% of an AD cohort and 69% of the age-matched normal group using enzyme-linked immunosorbent assay (ELISA) testing [48]. This data confirms the aforementioned PCR finding that HSV-1 DNA sequences are present in many elderly brains as a whole functional HSV-1 genome and provides evidence that the virus replicates in the brain [48].

HSV-1 IN THE BRAIN OF *APOE-ε*4 ALLELE CARRIERS INCREASES THE RISK FOR AD

Additional evidence for HSV-1 in AD involves the type-4 allele of the apolipoprotein E gene, known as *APOE-ε*4 or APOE4. A significantly increased risk for sporadic AD is associated with the presence of both HSV-1 in brain and carriage of the *APOE-ε*4 allele [49]. As shown in a study of AD

Table 1
Studies that have detected HSV-1 DNA using PCR in brain tissue from patients with AD and controls (non-neurological cases)

Study	Primers used for PCR	Area of brain sample	HSV-1 DNA-positive individuals	
			AD *n* (%)	Controls *n* (%)
Jamieson *et al*. [4]	TK	Temp, frontal cortex, hippocampus	8 (100)	6 (100)
Jamieson *et al*. [42]	TK	Temp, frontal cortex, hippocampus	21 (67)	15 (60)
Baringer and Pisani [271]	Various	Various	NR	40 (35)
Gordon *et al*. [272]	Various	Hippocampus and frontal cortex		30 (27)
Itabashi *et al*. [45]	gD	Temporal and frontal cortex	46 (30)	23 (22)
Itzhaki *et al*. [49]	TK	Frontal and temporal cortex	46 (67)	44 (64)
Lin *et al*. [50]	TK	Frontal and temporal cortex	61 (74)	48 (63)
Bertrand *et al*. [273]	gD	Various	98 (75)	57 (72)
Cheon *et al*. [274]	gD	Frontal cortex	10 (100)	10 (100)

HSV-1, herpes simples virus type 1; AD, Alzheimer's disease; PCR, polymerase chain reaction; gD, glycoprotein D protein; TK, thymidine kinase; NR, not Reported. Table adapted from Itzhaki R (2004) Herpes simplex virus type 1, apolipoprotein E and Alzheimer' disease. *Herpes* **11**(Suppl 2), 77A-82A. [41] Reprinted with permission from Ruth Itzhaki.

Table 2
APOE genotypes of Alzheimer's disease patients and aged non-Alzheimer's disease patients positive or negative for Herpes Simplex Virus Type 1 in all brain regions

	Overall data for all brain regions					
	Non-AD ($n=44$)			AD ($n=46$)		
	HSV1+	HSV1–	Total	HSV1+	HSV1–	Total
Genotype						
ε2/ ε2	0	0	0	0	0	0
ε2/ ε3	1	3	4	2	1	3
ε2/ ε4	0	0	0	0	0	0
ε3/ ε3	25	11	36	5	7	12
ε3/ ε4	2	2	4	20	2	22
ε4/ ε4	0	0	0	9	0	9
Allele number						
ε2	1	3	4	2	1	3
ε3	53	27	80	32	17	49
ε4	2	2	4	38	2	40
APOE- ε4	3.6%	6.3%	4.5%	52.8%	10.0%	43.4%

HSV-1 in brains of *APOE*-ε4 allele carriers accounts for over 50% of AD brains with testing done postmortem. Table from Itzhaki RF, Lin WR, Shang D, Wilcock GK, Faragher B, Jamieson GA (1997) Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* **349**, 241-244 [49]. Copyright 1997. Reprinted with permission from Elsevier and Ruth Itzhaki.

postmortem brains by Itzhaki *et al*. [49] and confirmed by Lin *et al*. [50], neither HSV-1 nor the *APOE*-ε4 allele alone was found to be a risk factor for AD. However, the combination of HSV-1 with the *APOE*-ε4 allele increased the risk for AD by a factor of 12 [50]. HSV-1 in the brains of *APOE*-ε4 allele carriers accounted for over half of AD patients in the study (Table 2) [49]. The proportion of HSV-1 positive elderly controls was similar to that of HSV-1 positive AD patients, indicating that the AD brain is not predisposed to HSV-1 infection. Few HSV-1 positive elderly controls were positive for the *APOE*-ε4 allele, indicating that *APOE*-ε4 allele carriers are not predisposed to HSV-1 infection [49]. Itzhaki's results were later confirmed by Itabashi and colleagues [45].

APOLIPOPROTEIN E INFLUENCES HSV-1 VIRAL LOAD IN ANIMAL BRAIN STUDIES

Apolipoprotein E dosage and the presence of *APOE*-ε4 determine latent HSV-1 DNA concentrations in the mouse brain [51]. Burgos and colleagues inoculated mice with HSV-1 and measured brain viral DNA concentrations. Thirty-seven days after infection, the HSV-1 brain DNA concentrations for APOE+/+ wild-type mice were 13.7 times greater than those of APOE–/– knockout mice. HSV-1 brain DNA concentrations for human APOE4 transgenic mice were 13.6 times greater than those of APOE3 mice. Apolipoprotein E4 appeared to facilitate HSV-1 latency in the brain much more than apolipoprotein E3, and APOE dosage correlated directly with the concentration of HSV-1 in the brain [51]. Guzman-Sanchez and collaborators later confirmed that apolipoprotein interacts with HSV-1 in animal models to increase viral load in the brain. 2-month-old wild-type and APOE knock-out mice were infected with HSV-1 and followed for 16 months. Viral load was found to increase with age. Viral load in the brains of aged APOE+/+ wild-type female mice was 43 times that seen in knock-out APOE–/– male mice. Although no neuropathological or brain MRI morphological differences were detected between 18-month-old infected mice when compared to controls, the central nervous system (CNS) HSV-1 infected mice showed associated memory deficit and reduction in metabolic indicators of CNS health [52]. These animal studies which associate APOE4 with increased HSV-1 viral load in the brain may relate to Itzhaki's human postmortem study, indicating that the combined presence of HSV-1 in brain and carriage of the *APOE*-ε4 allele are involved in the pathogenesis of AD [49].

GENOME-WIDE ASSOCIATION STUDIES

Two genome-wide association studies identified APOE, complement receptor 1 (CR1), clusterin (CLU), and phosphatidylinositol binding clathrin assembly protein (PICALM) as major susceptibility genes in AD [53]. These susceptibility genes are

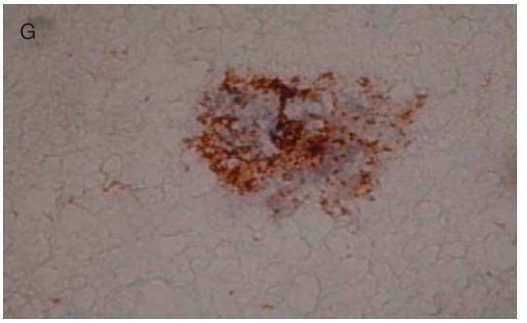

Fig. 1. Co-localization of HSV-1 DNA and amyloid-β in AD plaques. A strong co-localization showing HSV-1 DNA (brown staining using PCR) and amyloid plaque (blue staining using immunohistochemistry) in a postmortem AD brain (G). Greater than 90% AD plaques contained viral DNA. Scale bar = 50 μm. Figure from Wozniak, MA, Mee AP, Itzhaki RF (2009) Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. *J Pathol* **217**, 131-138 [3]. Copyright 2008. Reprinted with permission from John Wiley and Sons, Inc. and Ruth Itzhaki.

associated with the HSV life cycle, and relate either directly or indirectly to cellular entry, intracellular transport, nuclear egress, AβPP processing, and Aβ processing [53].

AD AMYLOID PLAQUES CONTAIN HSV-1 DNA

HSV-1 coexists with Aβ in AD amyloid plaques [3]. Using *in situ* PCR to detect HSV-1 DNA and immunohistochemistry or thioflavin S staining to detect amyloid plaques, Wozniak and coworkers discovered a striking co-localization of HSV-1 DNA and Aβ within senile plaques in postmortem brains (Fig. 1) [3]. In AD brains, 90% of the plaques contained HSV-1 DNA and 72% of the total brain HSV-1 DNA was associated with plaques. The HSV-1 DNA associated with plaques was much lower in aged normal brains than in AD brains ($p < 0.001$) [3]. The co-localization of HSV-1 DNA and Aβ within amyloid plaques in the AD brain places HSV-1 in direct juxtaposition with a highly significant AD biomarker and suggests a significant role for HSV-1 in AD pathogenesis.

IN VITRO AND ANIMAL STUDIES: HSV-1 INFECTION INDUCES ELEVATED LEVELS OF Aβ AND P-TAU

Human cultured neuroblastoma cells infected with HSV-1 *in vitro* produce $A\beta_{42}$ and $A\beta_{40}$, and increased amounts of the enzymes β-site AβPP-cleaving enzyme (BACE-1) and nicastrin (a component of the γ-secretase enzyme) [10]. Both enzymes are involved in cleavage of the AβPP to produce Aβ. Rapid reduction of AβPP and a dramatic increase in $A\beta_{42}$ and $A\beta_{40}$ is seen in HSV-1-infected neuronal cell cultures [10]. Rat cortical neurons challenged with HSV-1 demonstrate hyperexcitability, membrane depolarization, and increased intracellular calcium levels with enhanced calcium dependent-AβPP phosphorylation and intracellular accumulation of $A\beta_{42}$ [54]. Animal models also support HSV-1 as causative in the formation of Aβ. Mouse brain infected with HSV-1 produced marked increases in $A\beta_{42}$ five days post-intranasal infection when compared to uninfected controls [10]. These studies indicate that HSV-1 exposure to neuronal cells results in cellular production of Aβ.

HSV-1 is able to induce tau phosphorylation, thus linking HSV-1 to the formation of another abnormal protein found in AD brains. Neuroblastoma cells infected with HSV-1 produce hyperphosphorylated tau protein and increased amounts of the enzymes that phosphorylate tau protein including glycogen synthase kinase-3β (GSK-3β) and protein kinase A [11]. Alvarez *et al*. demonstrated accumulation of hyperphosphylated tau protein within the nucleus of HSV-1 infected neuroblastoma cells [55]. Zambrano *et al*. showed that HSV-1 infection of murine neuronal cultures results in tau hyperphosphorylation and alterations in the microtubule dynamics of the neuronal cytoskeleton [56]. The ability of HSV-1 to induce phosphorylation of tau proteins in neuronal cells is significant because p-tau proteins contribute to the formation of NFTs in AD brains [2].

ADDITIONAL MOLECULAR EVIDENCE AND CELLULAR MECHANISMS RELATING HSV-1 TO AD

Additional studies show a structural link between HSV-1 and Aβ. $A\beta_{34\text{-}42}$ is 67% identical to the HSV-1 envelope protein glycoprotein B (gB) peptide sequence, indicating peptide homology [57]. Synthetic peptides derived from the HSV-1 gB fragment self-assemble into thioflavin-positive fibrils and form β-pleated sheets that are ultrastucturally indistinguishable from Aβ [57]. The gB fragment accelerates *in vitro* formation of Aβ fibrils that are toxic to

primary cortical neurons at a dose comparable to Aβ [57].

HSV-1 travels inside the neuronal cytoplasm in association with AβPP [58]. In squid axons, HSV-1 travels with AβPP during fast anterograde transport from the nerve cell body down the axon [59]. The virus interferes with AβPP processing in HSV-1-infected neuronal cells, reducing the level of AβPP and increasing the level of a 55 kDa C-terminal AβPP fragment containing Aβ [60]. De Chiara *et al*. found that HSV-1 infection of neuroblastoma cells and rat cortical neurons induces multiple cleavages of AβPP with resultant neurotoxic intra and extracellular AβPP fragments that comprise portions of Aβ. Components of the amyloidogenic AβPP processing pathway, including host cell β-secretase, γ-secretase, and caspase-3 like enzymes, were shown to be involved in the AβPP cleavage process. These findings suggest that repeated HSV-1 reactivation in the presence of other risk factors may play a co-factorial role in the development of AD [61]. Cheng and colleagues evaluated HSV-1 interactions with AβPP using immune-fluorescence, immunogold electron microscopy, and live cell confocal imaging to visualize newly synthesized viral particles inside epithelial cells as they traveled to the cell surface. Cytoplasmic HSV-1 particles labeled with green fluorescent protein co-localized and traveled with AβPP inside living cells. Most intracellular HSV-1 particles interacted frequently with AβPP, which facilitated viral transport while interfering with normal AβPP transport and distribution. Intracellular HSV-1 interactions with AβPP provide a mechanistic basis for the association between HSV-1 seropositivity and AD [62].

Santana *et al*. demonstrated that HSV-1 infection of neuroblastoma cells induced significant intracellular accumulation of Aβ in autophagosomes and a marked decrease in Aβ secretion. Aβ failed to fuse with lysosomes in HSV-1-infected neuroblastoma cells, indicating the impaired degradation of Aβ localized in autophagic vesicles [63]. HSV-1 decreases autophagy using HSV-1 infected cell polypeptide 34.5 (ICP 34.5) which blocks the protein kinase R (PKR) and eukaryotic initiating factor 2α (eIF2α) signaling pathways [64]. This action inhibits HSV-1 degradation by interfering with autophagy of the virus [64, 65]. Itzhaki suggests that this may lead to a decrease in Aβ clearance and an accumulation of senile plaques in AD [66].

HSV-1 can both block and induce neuronal apoptosis. HSV-1 protein ICP34.5 dephosphorylates eIF2α to block both the shutdown of host cell protein synthesis and apoptosis [66, 67]. HSV-1 infection of murine neuronal cultures results in marked neurite damage and neuronal apoptosis [56].

ADDITIONAL MOLECULAR STUDIES LINKING HSV TO AD

Amyloid-β precursor protein (AβPP) is an integral cell membrane glycoprotein. Within the amyloidogenic pathway, AβPP undergoes cleavage by β-secretase, producing the N-terminal soluble fragment sAPPβ. The enzyme γ-secretase then cleaves the intramembranous APP C-terminal fragment (CTFβ) to form Aβ and APP intracellular domain (AICD) [278]. Civitelli *et al*. found that infection of both rat cortical neurons and human neuroblastoma cells *in vitro* by HSV-1 induced AβPP amyloidogenic processing [279]. Specifically, HSV-1 infection induced the formation of APP intracellular domain (AICD) which accumulated in the nucleus of infected cells. AICD bound the promoter region of the neprilysin genes NEP*prom*1 and NEP*prom*2, causing a transient increase in mRNA levels followed by a reduction in *nep* mRNA, protein, and enzymatic activity. Neprilysin is a major Aβ-degrading enzyme in the brain [280]. AICD also bound the promotor region of the glycogen synthase kinase-3β (*gsk3*β) gene. Gsk3β protein expression remained unchanged; however, HSV-1 modulated gsk3β enzymatic activity through phosphorylation of gsk3β in the late stages of viral infection [279]. The enzyme GSK3β is involved in tau hyperphosphorylation and Aβ production [281]. This study demonstrates mechanisms whereby HSV-1 infection induces upstream events in neuronal cells which affect enzyme systems involved in Aβ production and clearance, as well as tau protein hyperphosphorylation in the AD brain [279].

D'Aiuto *et al*. studied glutamatergic neurons derived from human induced pluripotent stem cells infected with HSV-1 [282]. Microarray analysis revealed that during the lytic phase, viral infection caused extensive changes in neuronal gene expression that affected both cAMP response element-binding protein (CREB) and glutamate signaling. During quiescent infection induced by antiviral drugs, HSV-1 affected neuronal gluta-

mate receptor genes and voltage-gated ion channel genes [282]. These HSV-1 related effects involving cognition-related pathways are relevant because lower levels of CREB [283], glutamate excitotoxicity [284], and abnormalities related to voltage-gated ion channels [285, 286] have been implicated in AD and cognitive impairment.

D'Aiuto *et al*. also evaluated HSV-1 seropositive schizophrenic and non-psychiatric subjects using working memory cognitive testing with concurrent functional MRI (fMRI) [282]. They found that during testing, HSV-1 exposed subjects had significantly increased hemodynamic responses in the frontoparietal, thalamus, and midbrain regions, regardless of schizophrenic diagnosis, compared to HSV-1 seronegative controls. None of the subjects had a history of HSV encephalitis. The fMRI results suggest that increased processing time for working memory performance is associated with HSV-1 exposure [282]. The data from D'Aiuto *et al*. provides a potential mechanistic link between HSV-1 infection and cognitive impairment through altered gene expression and subsequent neuronal dysfunction [282].

Studies have shown that HSV-1 infection affects synaptic transmission. HSV-1 infection of mouse cortical neurons results in reduced levels of presynaptic proteins synapsin-1 and synaptophysin. Inhibition of cAMP response element-binding protein (CREB) and decreased synaptic transmission was also demonstrated [287]. Decreased levels or activity of these synaptic proteins have been found in the AD brain [283, 288, 289]. These molecular abnormalities and associated synaptic dysfunction were shown to be mediated by HSV-1 induced glycogen synthase kinase-3 (GSK-3) activation and intraneuronal accumulation of Aβ [287]. HSV-1 induced alterations in levels of synaptic proteins and synaptic dysfunction support the hypothesis that HSV-1 infection of the central nervous system is a contributing factor in the pathophysiology of AD.

HSV-2 has been found in AD and normal elderly postmortem brains, although at a relatively low prevalence compared to HSV-1 [88]. Kristen *et al*. found that human neuroblastoma cells infected with HSV-2 *in vitro* demonstrated abnormal APP proteolytic processing, impairment of autophagy, and intracellular Aβ accumulation [290]. HSV-2 infection also resulted in tau hyperphosphorylation. This study is the first to demonstrate that in neuronal cells, HSV-2 infection induces similar pathophysiologic processes and AD-like markers of neurodegeneration as HSV-1 infection [10, 11, 60, 63, 64, 290].

Aβ ACTS AS AN ANTIMICROBIAL PEPTIDE (AMP) WITH ANTI-VIRAL ACTIVITY AGAINST HSV-1

Bourgade *et al*. found that $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ inhibit HSV-1 replication when Aβ is added 2 hours prior or concomitantly with HSV-1 infection of fibroblasts, epithelial, or neuronal cells [291]. Aβ did not display anti-viral activity against non-enveloped human adenovirus. The authors conducted a series of experiments using a cell-free system with fluorescence detection assays. Based on this data, they propose that Aβ peptide interacts with the HSV-1 envelope in the extracellular environment, resulting in decreased attachment and/or fusion with host cell membranes. Aβ shares peptide homology with HSV-1 envelope glycoprotein B (gB) [57], leading the authors to suggest that the Aβ effect on HSV-1 replication may involve its insertion into the HSV-1 envelope [291].

The same group found that $A\beta_{42}$ showed anti-viral AMP activity against HSV-1 in an *in vitro* co-culture model consisting of neuroglioma and glioblastoma cells [292]. Neuroglioma cells infected by HSV-1 produced $A\beta_{42}$, which was then internalized by glioblastoma cells. Conditioned medium from HSV-1 infected neuroglioma cells conferred Aβ-dependent protection against HSV-1 replication in *de novo* neuroglioma cells challenged with HSV-1 infection [292]. The authors hypothesize that Aβ peptides are antimicrobial peptides produced by neuronal cells under homeostatic conditions to fight viral infections such as HSV-1. Overproduction of Aβ in response to HSV-1 infection, other infections, or additional pathological insults results in formation of fibrillar Aβ, amyloid plaques, and neuronal damage, which contributes to neurodegeneration and AD [292].

HSV-1 INDUCES AD-LIKE INFLAMMATION AND OXIDATIVE STRESS

Elevated levels of pro-inflammatory cytokines are consistently found in the brains of AD patients [19, 20]. Infection by HSV-1 induces expression of cytokines and pro-inflammatory molecules, including interleukin-1β (IL-1β), tumor necrosis factor-α

(TNF-α), IL-6, IL-8, macrophage inflammatory protein 1-α (MIP-1 α), chemokine (C-C motif) ligand 5 (CCL5), and chemokine CXCL 10 in human microglial cells [17]. Persistent cytokine expression occurs in mouse trigeminal ganglion infected with HSV-1, including IL-2, IL-6, TNF-α, interferon- γ (IFN- γ), IL-10, and CCL5 [18]. The direct effects of HSV-1 on neurons and the host inflammatory response to infection can lead to oxidative damage due to increased formation of reactive oxygen and reactive nitrogen species [68].

Interactions between HSV-1 and oxidative stress promote neurodegenerative processes found in AD. In HSV-1 infected human neuroblastoma cells, experimentally induced oxidative stress was found to significantly enhance the accumulation of intracellular Aβ, inhibit Aβ secretion, and appeared to be mediated by HSV-1 infection [69]. Oxidative stress also potentiated the accumulation of autophagic compartments within the cell [69]. HSV-1 interactions with oxidative stress are significant because oxidative damage is thought to occur early in the pathogenesis of AD [70].

HSV-1 REACTIVATION IN THE BRAIN

Itzhaki points out the lack of methodology for detecting the hypothesized sub-clinical limited reactivation of HSV-1 in localized areas of the brain in AD patients [32]. This contrasts with clinically apparent acute HSV encephalitis, where detection of HSV-1 DNA in cerebrospinal fluid (CSF) is commonly used for diagnosis [32]. Mild forms of HSV-1 encephalitis in humans have been reported [71, 72]. These patients usually have less severe symptomatology and good prognoses when compared to patients with severe diffuse HSV-1 meningoencephalitis. Klapper *et al*. suggest that sub-acute HSV-1 encephalitis may be a more common and often missed sub-clinical presentation of the disease [71].

Peter and collaborators reviewed 3,200 randomly selected CSF specimens submitted for HSV testing and found a total of 62 HSV positive specimens. HSV-2 was detected more often than HSV-1 (36:26). However, the HSV-2: HSV-1 ratio reversed in the patients over age 60 with HSV-1 being more prominent (3:13). Female patients who were positive for HSV-1 predominated in the over-70 age group (10 female and 1 male with 90% of females positive for HSV-1). This study shows predominance in the reactivation of HSV-1 rather than HSV-2 in older females, a group known for having a higher incidence of AD [73].

Saldanha *et al*. found that HSV-1 reactivates in the brains of immunosuppressed patients. HSV-1 DNA was detected by *in situ* hybridization in postmortem frontal and temporal lobe human brain samples from immunosuppressed leukemia patients who were seropositive for HSV-1. HSV-1 DNA was not found in HSV-1 seronegative patients or in those who had not been immunosuppressed [74].

The reactivation rate of HSV-1 in the human brain is not known; however, animal and human studies involving the CNS and peripheral nervous system suggest that periodic sub-clinical reactivation may occur with subsequent immune response and neurodegeneration. Kaufman *et al*. measured the rate of asymptomatic HSV-1 reactivation and shedding in human tears from normal adults without signs of ocular herpetic disease. 74% of the 50 subjects were positive for HSV IgG by ELISA. 49/50 (98%) of subjects shed HSV-1 DNA at least one time during the course of the 30-day study [75].

Margolis and colleagues described spontaneous molecular reactivation of HSV-1 with viral protein expression, positive HSV-1 antigen staining, and infectious virus found in 6% of "latently" infected murine sensory ganglia 37 days post ocular infection [76]. Using *in situ* hybridization and immunohistochemistry, Feldman *et al*. found that HSV-1 spontaneous reactivation occurred in one neuron per 10 HSV-1 latently infected mouse trigeminal ganglia tested [77]. These neurons were surrounded by focal white cell infiltrate, indicating an inflammatory response. The authors estimate that this is equivalent to one neuron expressing high-level productive cycle viral genes in each ganglion every 10 days [77].

Asymptomatic reactivation of HSV-1 occurs *in vivo* in the CNS of mice and is associated with production of markers of neurodegeneration found in AD [78]. HSV-1 reactivation from the asymptomatic latent phase, was demonstrated by detection of viral ICP4 protein in the trigeminal ganglion and cerebral cortex of mice 60 days post-infection. Reactivation was accompanied by upregulation of both markers of neuroinflammation [(toll-like receptor (TLR)-4, interferon α/β, and phosphorylated interferon regulatory factor 3 (p-IRF3)] and early neurodegeneration (phospho-tau and caspase-3 cleaved tau proteins) [78]. These findings support the hypothesis that recurrent HSV-1 CNS reactivation could result in AD associated neurodegenerative processes.

PROPOSED MECHANISM OF HSV-1 PATHOGENESIS IN AD

The above data and studies support the hypothesis that HSV-1 in combination with *APOE*-ε4 allele carriage is a major cause of sporadic AD. As proposed by Itzhaki, this highly prevalent virus reactivates and enters the brain in older age by way of the peripheral nervous system or the olfactory route. HSV-1 becomes latent in the brain, but periodically reactivates in association with systemic infection, immunosuppression, or other stressors. The reactivated virus causes limited local damage via direct viral action and through inflammatory and oxidative effects. This leads to deposition of Aβ and abnormal phosphorylation of tau, which eventually forms amyloid plaques and NFTs. Defective autophagy due to aging and viral ICP34.5 action prevents degradation of HSV-1 and reduces the degradation of Aβ and phosphorylated tau protein. This results in decreased clearance of these proteins [66, 79, 80].

CYTOMEGALOVIRUS AND OTHER *HERPESVIRDAE*

Cytomegalovirus

CMV is a β-herpes virus prevalent in humans causing persistent lifelong asymptomatic infection in the immunocompetent host. Primary infection usually occurs early in life and is asymptomatic but occasionally causes a self-limiting mononucleosis-like syndrome [81]. CMV seropositivity in the human population ranges from 20%–100% depending on socioeconomic status and age [82]. The virus resides in the myeloid cell compartment, remaining latent in monocytes [83], but has tropism for numerous cell types such as endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, neuronal cells, hepatocytes, trophoblasts, macrophages, and dendritic cells [81]. As with other members of the *Herpesviridae* family, CMV may reactivate under stress conditions or other stimuli. Other diseases associated with CMV infection in normal hosts include Guillain-Barre syndrome, meningoencephalitis, hemolytic anemia, and thrombocytopenia [81]. CNS infection by CMV in immunocompetent patients is rare. Most CMV brain infections occur in those who are immunocompromised, such as HIV-infected patients, transplant recipients, and infants with congenital CMV disease contracted *in utero* [82].

Epidemiological studies: CMV humoral response, cognitive decline, and AD

Several studies have shown an association between CMV infection and increased risk of both cognitive impairment and development of AD. Aiello *et al*. found that individuals with higher levels of IgG antibody to CMV at baseline experienced a more rapid rate of cognitive decline over a 4-year study period than those with lower levels [84]. Strandberg *et al*. [26] and Katan *et al*. [27] found that CMV was one of the viruses from the *Herpesviridae* family associated with cognitive decline as discussed in the HSV-1 section above. Carbone *et al*. studied a group of elderly patients and found baseline CMV IgG antibody levels to be significantly increased in patients who developed clinical AD over a 5-year follow-up period compared to patients who remained cognitively healthy [85]. Barnes *et al*. followed 849 participants and found that baseline CMV seropositivity doubled the risk of developing clinical AD over a 5-year follow-up period and noted a faster rate of decline in global cognition [86].

Tarter *et al*. studied cognitive impairment in various age groups in relation to CMV and HSV-1 seropositivity. Among children (ages 6–16 years), HSV-1 seropositivity was associated with lower reading and spatial reasoning test scores. Both HSV-1 and CMV seropositivity in middle–aged adults (ages 20–59 years) was associated with impaired coding speed. CMV seropositivity was also associated with impaired middle-aged learning and recall. Among older adults, HSV-1 seropositivity was associated with immediate memory impairment. The data indicated that HSV-1 may have a life course effect on cognition across all age groups, while CMV appeared to adversely affect cognition specifically in the middle aged. The authors suggest that individuals who acquire infection with these *Herpesviridae* earlier in life with more reactivations and subsequent immune activation may be at greater risk for developing social disparities in cognition, educational attainment, and social mobility across the life course [87].

Prevalence of CMV in the AD Brain

Data does not indicate a definitive direct infiltrative CNS role for CMV in AD. Using PCR, Lin *et al*. found CMV present in 16/45 (35.6%) of postmortem AD brains compared with 10/29 (34.5%) of non-AD controls, which was not statistically significant [88]. The authors point out that these values may be artificially high due to the possibility of CMV residing in

lymphocytes within blood vessels rather than brain cells. In a more recent 2013 study, 93 AD brains were tested for CMV DNA using nested PCR and all samples were negative for CMV [85]. In contrast, Lin *et al*. found CMV in a very high proportion of postmortem vascular dementia brains [89]. CMV was found in 14/15 (93%) of brains from subjects who had been diagnosed with vascular dementia and was present in only 10/29 (34%) of age-matched normal controls. The results were statistically significant and suggest a possible role for CMV in vascular dementia [89].

CMV and immunosenescence: Impairment of the elderly immune system

CMV appears to be a strong causative factor in the development of immunosenescence by adversely affecting T cell immunity with resultant immune dysregulation and impairment in the elderly [90]. CMV has been implicated in T cell oligoclonal expansions, altered phenotypes and function of CMV specific CD8+ T cells, and decrease in the naïve and early memory T cell pool seen in the elderly [90]. Koch *et al*. reviews evidence for CMV involvement in immunosenescence and suggests that the long-term attempt of the T-cell immune system to keep CMV from spreading results in reduction of the naïve T-cell pool, leading to deficits in the immunological response to new antigens in the aged [90]. Clonal expansions of CD8+ T cells directed against another Herpes virus, EBV, are also seen in the aged population [91].

Increased reactivation of CMV and other herpesviridae in the elderly

Using molecular and serological techniques, Stowe *et al*. found significant increases in reactivation of CMV and EBV in elderly subjects compared to younger subjects. Increases in CMV DNA in urine and Epstein Barr viral load in peripheral blood were demonstrated. In addition, elevated levels of CD8+ T cells directed against CMV and EBV were found in the elderly group. The authors concluded that the aged immune system is no longer able to control EBV and CMV reactivation, resulting in chronic infection and age-related clonal expansions of CD8+ T cells directed against EBV and CMV [91].

Evidence suggests that CMV infection may adversely influence the immune response, allowing for increased HSV-1 reactivation. Stowe *et al*. measured serum CMV and HSV-1 antibody levels in 1,454 multiethnic subjects. Higher HSV-1 IgG serum antibody levels, which presumably reflect higher rates of HSV reactivation, were more common in CMV seropositive subjects. Elevated antibody titers to latent HSV-l were significantly associated with both CMV seropositivity and high CMV antibody levels. Increases in HSV-1 antibodies by age occurred in CMV seropositive individuals but not CMV seronegative subjects. Among CMV seropositive subjects, increases in HSV-1 antibodies by age were only found in individuals with low CMV antibody levels, as those with high CMV antibodies already exhibited elevated HSV-1 antibodies. The results suggest chronic CMV infection is able to accelerate immunosenescence, leading to immune dysregulation with increased HSV-1 reactivation [92].

CMV is associated with elevated IFN-γ that associates with AD

CMV-specific CD8+ T cells have been shown to produce increased amounts of pro-inflammatory IFN-γ and very low levels of anti-inflammatory cytokines IL-2 and IL-4 with a potential shift to a pro-inflammatory cytokine profile in the elderly [93]. Westman *et al*. measured the cytokine response of peripheral blood mononuclear cells (PBMCs) from CMV seropositive and seronegative AD patients. PBMCs from CMV seropositive AD patients challenged by CMV antigens produced increased amounts of IFN-γ compared with CMV seronegative AD patients and CMV seropositive nondemented controls [94]. The authors suggest CMV acts as an inflammatory promoter in AD immunology.

In the Rush AD Center Religious Orders Study, Lurain and colleagues studied a clinical-pathological community cohort by evaluating CMV serum antibody levels, CSF IFN-γ levels, cryopreserved lymphocytes, and brain pathology from deceased and autopsied subjects [82]. CMV-specific serum IgG antibody levels were significantly associated with NFTs. CSF IFN-γ was detected in greater than 80% of CMV seropositive but not in CMV seronegative subjects. In the CMV seropositive subjects, CSF IFN-γ levels were associated with NFTs. Therefore, this study showed an association between CMV seropositivity and detection of IFN-γ in CSF, which in turn was associated with AD pathology in the form of NFTs. In addition, the percentage of senescent T cells (CD28- CD57+) from the peripheral circulation was significantly higher for CMV-seropositive as compared to CMV-seronegative subjects [82]. This study did not prove CMV presence in the brain of

AD patients as CMV intrathecal antibody levels were not measured, and thus viral replication of CMV within the brain was not substantiated [95]. However, results from the Lurain *et al*. study support an association between CMV infection and the development of AD with CMV-induced inflammation as one potential mechanism for this association [96].

Human herpesvirus 6

HHV-6 is a neurotropic virus and exists in 2 forms: type A and type B. The HHV-6A variant is considered more neurotropic than type B [97]. HHV-6B primary infection is the cause of the common childhood illness exanthem subitum, which is also known as roseola infantum or sixth disease. This illness affects infants and typically presents with self-limiting fever followed by a maculopapular rash. Febrile seizures occur in 10–15% of cases, and severe CNS complications have been reported in rare cases. The virus is highly seroprevalent, affecting nearly 100% of the population by age 3 [98]. HHV-6 can cause meningoencephalitis, and has been associated with multiple sclerosis, seizures, and temporal lobe epilepsy [99]. HHV-6 establishes latency in the brain and can reactivate under conditions of immunosuppression [97].

HHV-6 has been found in the brains of AD patients in various studies using PCR; however, increased incidence in AD patients versus controls has not been shown with consistent statistical significance. Lin and collaborators studied 50 postmortem AD brains and found HHV-6 in 72% of frontal and temporal cortex samples versus 40% of age-matched normal brain samples, which was statistically significant [88]. In the HHV-6 positive brains, 59% (17/29) had type B alone, 3% had type A alone, and 38% (11/29) had both types. No additional increased risk for AD was found in *APOE*-ε4 carriers who were HHV-6 positive. The authors reasoned that HHV-6 might enhance the damage caused by HSV-1 and *APOE*-ε4 in AD. However, it was not possible to exclude HHV-6 as an opportunistic secondary infection, or the possibility that HHV-6 DNA is present within leucocytes within the brain vasculature [88]. Hemling *et al*. examined autopsy brain samples from hippocampus, temporal cortex, frontal cortex, and anterior cingulate gyrus, and found HHV-6 in 88% of AD and 87.5% of normal controls, indicating no significant difference between the two groups. However, the number of specimens from the different brain regions tested was not specified [46]. Carbone and colleagues found HHV-6 in 17.3 % of frontal cortex samples from postmortem AD patients using qPCR with no *APOE*-ε4 carrier association found. The same group found that baseline HHV-6 DNA positivity in peripheral blood leukocytes (PBLs) was significantly associated with cognitive decline and development of AD at 5-year follow-up [85].

Epstein barr virus

EBV is a Herpes virus that infects 95% of humans early in life resulting in lifelong latent asymptomatic infection residing in B-lymphocytes [100]. Primary infection of the oropharynx often occurs during childhood and is generally asymptomatic, although the virus does cause acute infectious mononucleosis in a minority of immune competent subjects. Intermittent reactivation of the virus occurs throughout life within B cells, involving a lytic cycle at mucosal sites with low levels of asymptomatic viral shedding [101]. EBV is causatively linked to Hodgkin lymphoma, Burkitt lymphoma, and nasopharyngeal carcinoma [102, 103]. EBV is also associated with neurological diseases including encephalitis, myelitis, mononeuritis [104,105], and multiple sclerosis [106].

Although EBV-related AD data is limited, the virus may be a risk factor for development AD. Carbone *et al*. measured EBV DNA in PBLs and postmortem brain samples from a group of AD subjects and non-AD controls. 45% of PBLs were EBV DNA positive in AD patients compared to 31% of controls, which was statistically significant. Using qPCR, only 6% of AD brains were EBV DNA positive with all of these subjects found to be *APOE*-ε4 positive. The same researchers found that baseline EBV DNA positive PBLs and serum IgG levels for EBV antigens were significantly increased in a group of elderly individuals who developed AD during a subsequent 5-year follow-up period [85]. Thus, positive IgG levels for EBV and peripheral viral infection involving PBLs with either EBV or HHV-6 have been associated with increased risk for AD even though significant infiltrative CNS presence has not been demonstrated for EBV and has been equivocal for HHV-6.

Bacterial pathogens

Chlamydophila pneumoniae

C. pneumoniae is an obligate intracellular respiratory pathogen that can persist as a chronic infection

in monocytes, macrophages, and other cell types for long periods of time. Serum antibody prevalence reaches 70% to 80% by 60 to 70 years of age [107]. Evidence indicates that *C. pneumoniae* crosses the blood-brain barrier (BBB) after infection of the respiratory mucosa, with subsequent hematogenous and lymphatic dissemination within infected monocytes [108, 109]. *C. pneumoniae* is also thought to enter the CNS via the olfactory route [80]. *C. pneumoniae* can evade the mechanisms of bactericidal and oxidative stress, activate endothelial cells with production of adhesion molecules, and induce cytokine overproduction [107].

C. pneumoniae has a biphasic life cycle. The elementary body is spore-like, infectious, metabolically inactive, and attaches to and enters the host cell. Elementary bodies then differentiate into reticulate bodies, which are the reproductive forms. The reticulate bodies undergo binary fission, differentiate back into elementary bodies, and exit the cell either by cytolysis with apoptosis or by exocytosis, leaving the cell intact [107, 110].

C. pneumoniae interferes with the normal apoptotic signaling pathways and can both inhibit and induce cellular apoptosis. The bacterium can evade the host cell's defense mechanisms, and exist as an acute infection or a chronic persistent infection [107, 111].

Under certain conditions, *C. pneumoniae* can enter into a chronic persistent phase characterized by aberrant reticulate bodies and other pleomorphic forms [112]. Metabolic activity is reduced and the organism is viable but non-cultivable, resulting in a chronic infection of the host cell [110, 112]. This persistent phase has been associated with several chronic diseases including asthma and chronic obstructive pulmonary disease [112].

Chlamydophila pneumoniae vascular infection and dissemination into the brain

Vascular infections with *C. pneumoniae* are associated with atheromasic plaques and may be an important factor in the development of brain infection with this pathogen. Using PCR and IHC techniques, Rassu *et al*. detected the presence of *C. pneumoniae* in atheromasic plaques sampled from five vascular sites in 18 autopsy cases (basilar artery, coronary artery, thoracic aorta, abdominal aorta, and renal arteries). The study showed 100% patient positivity with *C. pneumoniae* present at 2–5 sites for each case tested [113]. Di Pietro *et al*. investigated 19 postmortem cases with past chlamydial vascular infection using immunohistochemistry, PCR, *in situ* PCR and *in situ* reverse transcription PCR. *C. pneumoniae* was detected in the brain tissue of 16 out of 19 subjects (84.2%) who also had *C. pneumoniae* vascular infection. The organism was not detected in the brains of control subjects who were negative for *C. pneumoniae* vascular infection ($p = 0.0002$) [114]. These results provide evidence that a *C. pneumoniae* vascular infection can disseminate to the brain.

Prevalence of Chlamydophila pneumoniae in the AD brain

Balin *et al*. used PCR to identify *C. pneumoniae* in 17/19 (90%) of AD postmortem brain samples, and in only 1/19 (5%) of control brain samples, suggesting that infection with the organism is a risk factor for AD [6]. The results were confirmed using multiple methodologies. Electron microscopy and immunoelectron-microscopy studies identified chlamydial elementary and reticulate bodies in affected AD brain regions. *C. pneumoniae* was present in areas of the brain showing the typical AD neuropathology. Immunohistochemical tests on AD brains showed *C. pneumoniae* within pericytes, microglia, and astroglia. Reverse transcription (RT)-PCR assays using RNA from affected areas of AD brains confirmed the presence of transcripts from two important *C. pneumoniae* genes not seen in controls. Cultures were strongly positive for *C. pneumoniae* from a subset of affected AD brain tissues and negative in controls. *C. pneumoniae* was present, viable, and transcriptionally active in areas of neuropathology in the AD brain [6]. In addition to the standard morphological forms of the organism, pleomorphic forms of *C. pneumoniae* were later observed on ultrastructural analysis, suggesting an adaptive response and/or persistent state of infection for these organisms in AD [115]. As reviewed by Shima [116] and Balin [117], four studies [118–121] failed to detect significant *C. pneumoniae* in AD brains potentially due to sampling error, variable methodologies, and/or absence of standardized techniques.

Gerard and colleagues found *C. pneumoniae* in 20/25 (80%) of AD postmortem brain samples and 3/27 (11%) of controls (Fig. 2) [7]. Immunohistochemical analyses found that neurons, microglia, and astrocytes all served as host cells for *C. pneumoniae* [7]. Infected cells were seen in close proximity to senile neuritic plaques and NFTs [7]. In situ hybridization analysis in AD postmortem brains indicates an increase in the number of *C. pneumoniae* infected cells in *APOE*-ε4 carriers [123].

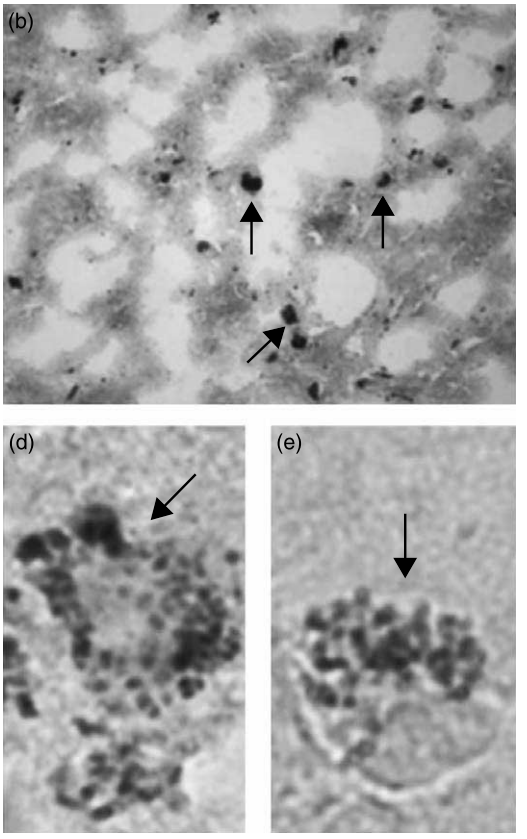

Fig. 2. Images demonstrating *Chlamydophila pneumoniae* in AD brain tissue by *in situ* hybridization. Figure (b) demonstrates *C. pneumoniae* from the hippocampus of an AD brain by *in situ* hybridization. Figures (d) and (e) show photographic enlargement of cells harboring *C. pneumoniae* inclusions identified in AD brain tissue. Arrows indicate the signal for C. *pneumoniae*. Image (b) was obtained using a x40 objective. Figure from Gérard HC, Dreses-Werringloer U, Wildt KS, Deka S, Oszust C, Balin BJ, Frey WH 2nd, Bordayo EZ, Whittum Hudson JA, Hudson AP (2006) Chlamydophila (Chlamydia) pneumoniae in the Alzheimer's brain. *FEMS Immunol Med Microbiol* **48**, 355-366 [7]. Copyright 2006. Reprinted with permission from John Wiley and Sons and permission from Brian Balin.

A statistically significant increase in CSF levels of *C. pneumoniae* DNA has been found in AD patients [122]. Miklossy found that combined data from studies attempting to isolate *C. pneumoniae* reached statistical significance in AD brains and AD brains plus CSF compared to controls (Table 3) [5].

Chlamydophila pneumoniae induces inflammation, Aβ plaque formation, and neurodegeneration

Increased levels of cytokines IL1-β, IL-6 and TNF-α were found in supernatants of *C. pneumoniae*-infected murine microglial cells *in vitro* [124]. Neurons exposed to the supernatants displayed a significant increase in apoptosis [124].

C. pneumoniae infection in the brains of BALB/c mice via the intranasal route induces a significant increase in Aβ plaques compared with non-infected mice [12]. Early treatment of infected mice with moxifloxacin decreased the number of Aβ plaques to levels similar to those seen in uninfected control mice. Infected untreated mice had 8-9 times more Aβ plaques than the antibiotic treatment group [117, 125].

Table 3
Detection of *Chlamydophila pneumoniae* in Alzheimer's disease

Material	Number	Method	AD	Control	Ref
Brain	38	PCR, EM, IHC, RT-PCR, Cult	17/19	1/19	[6]
Brain	25	PCR, IHC	0/25		[118]
Brain	20	PCR, IHC	0/20		[120]
Brain	20	PCR, Cult	2/15[a]	1/5[a]	[119]
Brain	21	PCR, ISH	21/21	0/1	[123]
Brain	52	PCR, Cult, RT-PCR	20/25	3/27	[7]
CSF	104	PCR, Cult	25/57	5/47	[122]
Total Brain	177	$p = 4.5 \times 10^{-7}$, OR = 8.7 CI = 3.1–29.5	60/125	5/52	
Brain and CSF	281	$p = 9.8 \times 10^{-11}$, OR = 7.8 CI = 3.7–17.8	85/182	10/99	

AD, number of AD cases with positive detection/number of AD cases analyzed: Control, number of control cases with positive detection/number of control cases analyzed; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; RT-PCR, reverse transcriptase-PCR; EM, electron microscopy; Cult, culture; P, exact value of significance following Fisher test; OR, odds ratio; CI, 95% confidence interval values; IHC, immunohistochemistry. [a]Positive in at least one of several samples. Table adapted from Miklossy J (2011) Emerging roles of pathogens in Alzheimer disease. *Expert Rev Mol Med* 13, e30 [5]. Copyright 2011. Reprinted with permission from Cambridge University Press and Judith Miklossy.

Spirochetes

Spirochetes are Gram-negative, helical bacteria that possess endoflagella. Spirochetes cause a number of chronic diseases including syphilis (*Treponema pallidum*), Lyme disease (Borrelia burgdorferi), and periodontal disorders such as gingivitis (oral periodontal Treponema spirochetes such as *T. sokranski* and *T. pectinovarum*) [5]. Spirochetes can invade the brain and form chronic persistent infections. They are known to spread by hematogenous dissemination, through the lymphatic system, and along nerve fibers [126].

Prevalence of spirochetes in AD brain

Spirochetes have been detected using various methodologies with prevalence approaching 90% in AD brains (Fig. 3) [5, 126]. The association was statistically significant in postmortem AD brain studies of all types of spirochetes combined, oral spirochetes, and *Borrelia burgdorferi*. Combined, studies detecting all types of spirochetes and their specific species indicated a prevalence of 68% (90/131) in AD brains compared to 8.45% in controls [5]. The spirochete frequency detected in all studies reviewed by Miklossy was eight times higher in AD brains than in controls [5]. Miklossy's extensive review of research data regarding spirochetes and AD indicate a probable causal relationship between neurospirochetosis and AD based on Koch's and Hill's criteria [126].

Using dark field microscopy, Miklossy identified spirochetes in blood, CSF, and brain in 14/14 AD autopsy cases and in 0/13 non-AD controls [9]. In this study, spirochetes were cultured from the blood of four AD cases. Concurrent silver stained and anti-AβPP-immunostained frozen section AD brain specimens evaluated with electron microscopy revealed spirochetes located in areas of AD pathology. Immunohistochemistry using a specific antibody against *B. burgdorferi* identified spirochetes in senile plaques, neurons, neuropil threads, and in the leptomeningeal and cortical vessel walls in a patient with concurrent Lyme disease and AD [9]. Electron microscopy and atomic force microscopy techniques have been used to identify spirochetes isolated and cultured from postmortem AD brains [8]. PCR and immunohistochemistry identified oral spirochetes in 14/16 AD and 4/18 non-AD postmortem brains (Fig. 4) [127]. DNA identified within neuropil threads in AD brains using the florescent dye DAPI revealed a helically shaped morphology similar to the morphology and distribution in reference spirochete samples [128]. Spirochetes were detected in the brains of 8/8 postmortem AD cases and in the blood samples from five living AD patients [129]. Using immunohistochemical techniques, Borrelia antigens (including the outer surface protein A (OspA) of *B. burgdorferi*) and Borrelia genes were co-localized with Aβ deposits and NFTs in three AD brains from which *B. burgdorferi* was cultured [130]. Bacterial peptidoglycan has been immunolocalized to senile plaques and NFTs in autopsied brain specimens from 54 AD patients [131, 132]. In addition, peptidoglycan and was found co-localized with Aβ in AD brains but not in controls [131]. The synthetic peptide BH (9-10), which corresponds to a β-hairpin segment of the *B. burgdorferi* OspA protein, forms amyloid-like fibrils *in vitro* [133].

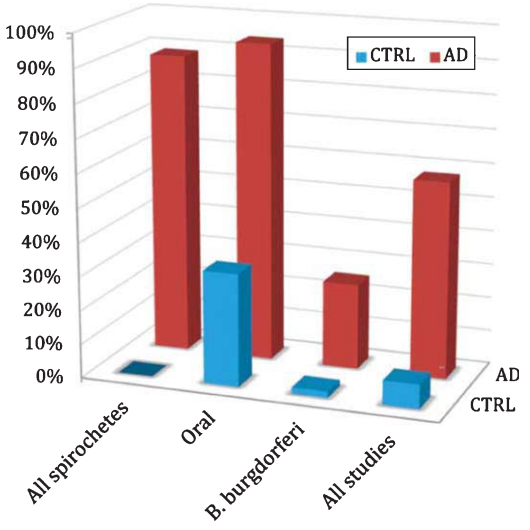

Fig. 3. Association of spirochetes with Alzheimer's disease. The frequency of spirochetes is significantly higher in the brains of Alzheimer's disease patients compared to controls. Graph from Miklossy J (2011) Alzheimer's disease - a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J Neuroinflammation* **8**, 90 [126]. Copyright 2011. Reprinted with permission under the terms of the Creative Commons Attribution License, (http://creativecommons.org/licenses/by/2.0) and permission from Judith Miklossy.

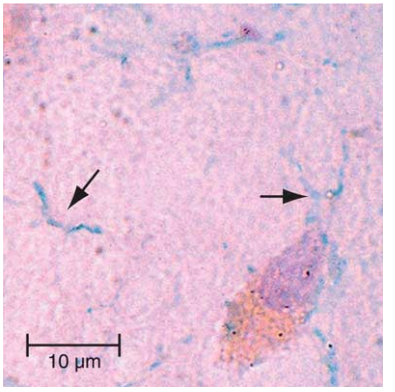

Fig. 4. Image of oral spirochetes in Alzheimer's disease brain. The oral spirochete *T. pectinovorum* stained dark blue (arrows) in a section from the hippocampus from an 84-year-old woman with Alzheimer's disease. The section was incubated with monoclonal antibodies to *T. pectinovorum*, and binding was disclosed using biotinylated anti-mouse antibodies and avidin-peroxidase. The photomicrograph was taken at1000X. Scale bar = 10 µm. Figure from Riviere GR, Riviere KH, Smith KS (2002) Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol Immunol* 17, 113-118 [127]. Copyright 2002. Reprinted with permission from John Wiley and Sons, Inc. and George Riviere.

B. burgdorferi induces Aβ and p-tau formation, inflammation, and neurodegeneration

In vitro, *B. burgdorferi* invades mammalian neurons and glial cells to cause an AD-like host cell reaction. Aβ deposition is induced *in vitro* by exposure of mammalian neurons, astrocytes, microglial cells, and brain organotypic cell aggregates to *Borrelia burgdorferi sensu strictu* [13]. Histochemical and immunohistochemical analysis showed morphological changes including Aβ plaques with β-pleated sheet conformation and tangles. Intracytoplasmic granules found in astrocytes were similar to the granulovacuolar degeneration seen in AD neurons [13]. Increases in AβPP, Aβ, and hyperphosphorylated tau proteins were detected by western blot in these cells [13]. Nuclear fragmentation in rat astrocyte cells exposed to pleomorphic and cystic forms of *B. burgdorferi* suggests that the spirochete can cause functional damage and apoptosis [134].

Exposure of rat glial cells to *B. burgdorferi* recombinant lipidated outer surface protein A (L-OspA) induces astrocyte proliferation and apoptosis. Astrocytes produce IL-6 and TNF-α in response to L-OspA [14]. *Ex vivo* stimulation of monkey brain explants with *B. burgdorferi* induces the production of cytokines IL-6, IL-8, IL-1β, cox-2, and the chemokine B lymphocyte chemoattractant (CXCL13) by glial cells, with concomitant glial and neuronal apoptosis [16].

Additional periodontal pathogens

In addition to the oral spirochetes discussed above, Kamer *et al*. found that both the number of positive tests for IgG serum antibodies against periodontal bacteria commonly involved in periodontitis (*A. actinomycetemcomitans, P. gingivalis*, and *T. forsythia*) and plasma TNF-α level were elevated in AD patients compared to normal controls. Both endpoints were independently associated with AD [135]. Results from the NHANES III study involving a large community sample, showed that the extent of periodontal disease, as measured by gingival bleeding, loss of periodontal attachment, and loss of teeth, was associated with significantly decreased cognitive function in early-, mid-, and late-adult life [136]. Cognitive testing included the Symbol Digit substitution and the Serial digit Learning Tests among patients 20–59 years of age and a Story Recall test in participants aged 70 years of age or older. Worse scores on all three measures of oral health status were significantly associated with poorer performance on all three measures of cognitive function after adjustment for age. Level of education was found to be an important confounding factor. The authors concluded that poor oral health is associated with impaired cognitive function throughout adult life [136]. A separate study from NHANES III showed an association between high serum antibody levels against *P. gingivalis* and lower cognitive function with delayed verbal recall and impaired subtraction in subjects greater than 60 years of age [137]. Thus, exposure to oral pathogens is associated with systemic inflammation, cognitive decline, and AD.

Helicobacter pylori

H. pylori is a curved spiral Gram-negative bacterium which colonizes the gastric mucosa in more than 50% of humans worldwide. The bacterium causes gastric disorders including functional dyspepsia, gastritis, peptic ulcer disease, and gastric cancer [138, 139]. *H. pylori* infection is associated with extra-digestive disorders including idiopathic thrombocytopenic purpura, vitamin B12 deficiency, and iron deficiency anemia [140]. The bacterium is also associated with vascular disorders such as atherosclerosis, ischemic stroke, and coronary artery disease [141].

H. pylori gastric infections may be diagnosed with non-invasive procedures including urea breath test, serology, or whole blood antibody testing depending on clinical circumstances [142]. Diagnostic tests for *H. pylori*, which require upper endoscopy with biopsy sampling of the gastric mucosa, include rapid urease test, histology, bacterial culture, and polymerase chain reaction technique [143].

Epidemiological studies: H. pylori infection, cognitive decline, and AD

Epidemiological studies support an association between *H. pylori* infection and both MCI and AD. Kountouras *et al*. studied sixty-three patients with amnestic MCI who underwent upper gastrointestinal endoscopy with histological and serological testing for *H. pylori* infection. Significantly increased *H. pylori* gastric infection, higher mean serum anti-*H pylori* IgG concentrations, and higher plasma total homocysteine titers were found in MCI patients compared to non-MCI anemic controls [144]. In another study, Kountouras and colleagues found a significantly higher rate of histologically proven *H. pylori* gastric infection among 50 AD patients compared to thirty non-AD anemic controls [145]. A longitudinal study by Roubaud-Baudron *et al*. followed 603 sub-

jects who were initially free of dementia and 65 years or older. Baseline seropositivity for *H. pylori* IgG antibody was associated with a 1.46 times increased risk for the development of dementia over the 20 year follow-up period compared to non-infected controls [146]. In a prospective non-randomized study, Kountouras *et al*. found that AD patients had significantly higher levels of anti-*H. pylori*-specific IgG antibodies in CSF and serum than age-matched cognitively normal controls. The severity of AD, as indicated by lower MMSE scores, correlated with higher levels of anti-*H. pylori* IgG antibodies in the CSF of these patients. The authors concluded that the data appears to link *H. pylori* infection to the pathophysiology of AD. They could not exclude the passage of *H. pylori* IgG and antibodies through an AD-related dysfunctional blood-CSF barrier to explain their findings [147].

In vitro and animal models: H. pylori induces formation of Aβ_{42} and P-tau

Mouse neuroblastoma N2a cells transfected with human AβPP are found to overexpress AβPP. Incubation of *H. pylori* filtrate with these cells resulted in increased production of presenilin-2 (a component of the gamma secretase enzyme complex) and Aβ_{42}. In the same study, intraperitoneal injection of *H. pylori* filtrate resulted in spatial learning and memory deficits in rats, abnormal hippocampal dendritic spine maturation, and increased presenilin-2 and Aβ_{42} in rat brain hippocampus and cortex [148].

H. pylori filtrate induced significant tau hyperphosphorylation at several AD-related tau phosphorylation sites in mouse neuroblastoma N2a cells through activation of glycogen synthase kinase-3β. In the same study, intraperitoneal injection of *H. pylori* filtrate in rats resulted in significant tau hyperphosphorylation in hippocampal areas of rat brain. Microglial activation and elevated brain/plasma cytokine levels were not found. The authors concluded that soluble exotoxins of *H. pylori* may induce tau hyperphosphorylation and that *H. pylori* eradication may be beneficial in the prevention of tauopathy [149]. These studies provide evidence which links *H. pylori* infection with AD-like Aβ and p-tau pathology.

Potential H. pylori pathogenic mechanisms in AD

Evidence for direct brain infiltration by *H. pylori* is lacking, and exactly how a gastrointestinal infection like *H. pylori* might influence neurodegeneration in AD is unknown. However, gastric inflammation has been found in patients infected by *H. pylori*, with increased production of IL-1, IL-6, IL-12, IL-18, TNF-α, and IFN-γ [150]. Lagunes-Servin *et al*. found that *H. pylori* gastric mucosa infection in children was associated with upregulation of toll-like receptors TLR2, TLR4, TLR5, and TLR9, and overproduction of cytokines, including TNF-α, IL-10, and IL-8 [151]. These findings are potentially significant because increased levels of pro-inflammatory cytokines and TLR-induced cell signaling cascades are implicated in AD pathogenesis [19, 20].

As reviewed by Kountouras and collaborators, additional proposed mechanisms for *H. pylori* related AD pathogenesis include: i) influences on neuronal apoptosis through molecular mimicry, in which homologous *H. pylori* epitopes induce humoral and cellular immune responses, which then cross-react with components of nerves; ii) molecular mimicry between *H. pylori* and endothelial antigens; iii) mononuclear cell production of a tissue factor-like pro-coagulant that converts fibrinogen into fibrin; iv) production of reactive oxygen species and circulating lipid peroxidases; v) platelet activation and aggregation; and vi) reduced levels of vitamin B12 and folate secondary to chronic atrophic gastritis, resulting in elevated serum homocysteine levels and subsequent endothelial damage and neurodegeneration [144, 152].

NEUROINFLAMMATION, PATHOGENS, AND NEURODEGENERATION

The innate immune system: Glial cells and the vicious cycle of inflammation

Gao and Hong [1] and Griffin [153] have advanced the hypothesis that uncontrolled inflammation drives neurodegenerative disease (Fig. 5) [1]. They propose that CNS pathological processes are initiated by environmental insults interacting with genetic susceptibility. Interactions between damaged neurons and deregulated, over activated microglia create a vicious self-propagating cycle causing uncontrolled long-term inflammation and progression of chronic neurodegenerative disease [1, 153].

Chronic overexpression of IL-1β is found in AD brains [20] and has been induced by pathogens *in vitro* [17,124] and *ex vivo* [16]. IL-1β has been shown to increase neuronal AβPP production [153, 154], apolipoprotein E levels, and astrocyte-mediated S100β protein levels [153]. BACE-1 levels in neurons are increased by cytokines [155], oxidative stress

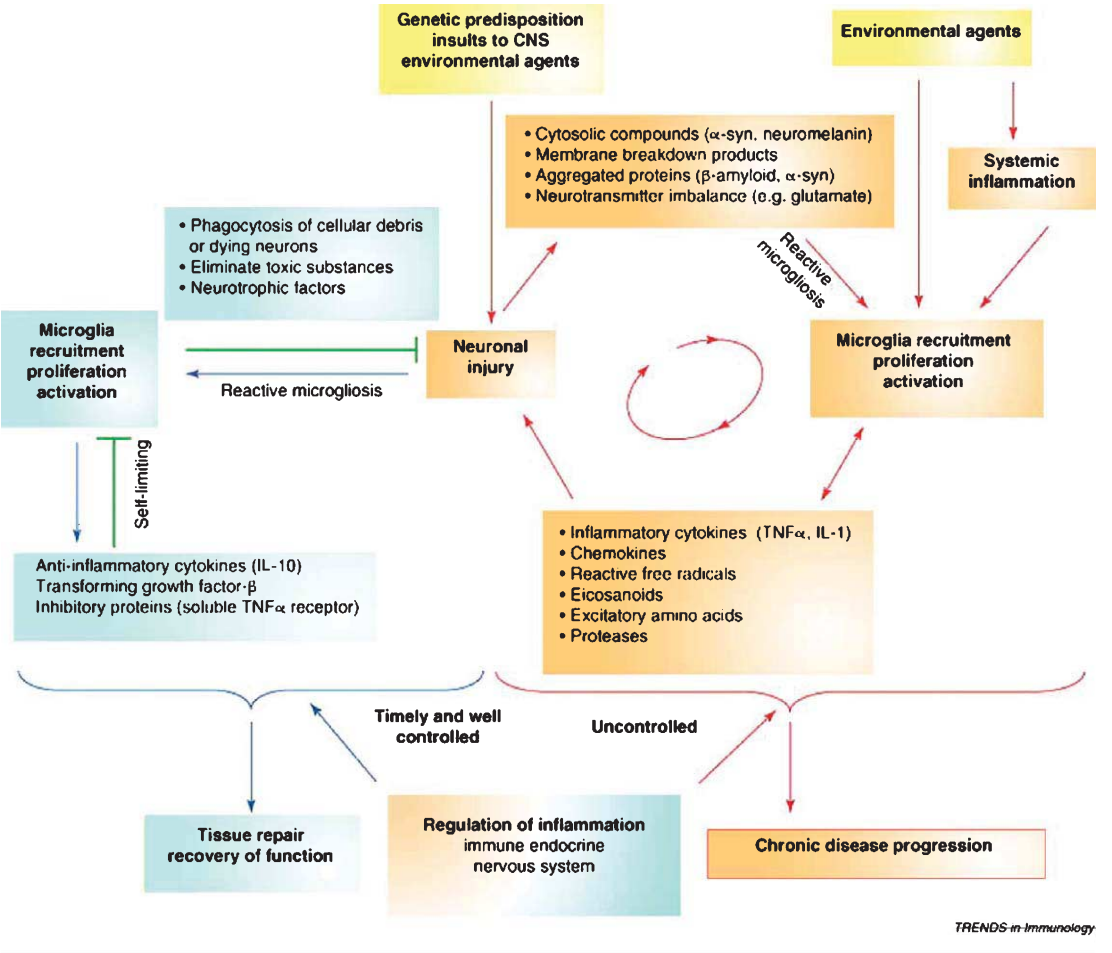

Fig. 5. The vicious cycle of neurodegeneration. Neuroinflammation when controlled is reparative and self-limiting but when uncontrolled forms a vicious cycle and leads to chronic neurodegeneration. Figure from Gao HM, Hong JS (2008) Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. *Trends Immunol* **29**, 357-365 [1]. Copyright 2008. Reprinted with permission from Elsevier and John Hong.

molecules [155], and pathogens such as HSV-1 [10]. Along with γ-secretase, BACE-1 catalyzes the conversion of AβPP to Aβ, resulting in elevated levels of toxic forms of Aβ [155]. Aβ activates microglial RAGE receptors, which appear to mediate the proinflammatory response to Aβ. Fibrillar Aβ activates microglia cell surface protein CD36 and scavenger receptors A and B (SR-A and SR-B). Activation of these receptors induces production of reactive oxygen species by microglia [156].

The cycle is further accelerated by neuronal injury and neuron cell membrane breakdown products, cytosolic compounds, and glutamate excess, which further activates microglia [1]. There is loss of homeostasis from a tightly controlled and regulated process where anti-inflammatory cytokines are utilized for tissue repair and recovery of function. The resultant uncontrolled inflammation and amplified cytokine cycle induces neuronal injury, apoptosis, and chronic disease progression [1, 153, 157].

Microglia function as innate immune cells in the brain [156]. Pattern recognition receptors located on the microglia cell surface identify pathogen associated molecular patterns on viruses and bacteria, leading to microglial production of pro-inflammatory molecules [5]. Pathogen associated molecular patterns include lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, flagellin, bacterial lipoprotein, and nucleic acid structures such as bacterial DNA or viral RNA [5]. Examples of pattern recognition receptors located on the microglia cell surface include TLRs1-9, scavenger receptors (SRA,

SRB, Macrophage receptor with collagenous domain (MARCO), CD36) and receptors for advanced glycogen end products (RAGE) [156].

Additional receptors include Major histocompatibility complex II (MHCII), cytokine receptors (CD40) and chemokine receptors (CCR3, CCR5) [20]. The NADPH oxidase receptor is a membrane-bound enzyme that catalyzes the production of superoxide from oxygen. This receptor is activated in the AD brain, and is associated with neurodegeneration [156]. Many of these receptors are upregulated in regions of typical AD brain pathology [19]. Increases in the levels of pattern recognition receptors in animal models and cell cultures are associated with neurodegeneration [19].

Microglia produce pro-inflammatory molecules via intracellular signaling pathways in cell cultures and animal models. For example, pathogens activate microglia TLRs leading to activation of nuclear factor κB, the mitogen activated protein kinases, and jun kinase. Pathogens can also induce activation of a second microglial pathway involving interferon regulatory factor-3 [5]. These pathways lead to the induction of inflammatory genes that produce cytokines and other pro-inflammatory compounds [5].

Microglia and astroglia are consistently found surrounding amyloid plaques in AD brains [157]. Amyloid deposition causes a microglial-mediated inflammatory response [19]. Pro-inflammatory molecules have been shown to be involved in pathways of neuronal apoptosis [20]. Aβ stimulated microglia secrete TNF-α and glutamate *in vitro*, resulting in simultaneous activation of neuronal TNF-α and N-methyl-D-aspartate (NMDA) receptors and subsequent neuronal apoptosis [158].

Pro-inflammatory compounds produced by glial cells and upregulated in AD brains include cytokines (IL-1α, IL-1β, IL-6, TNF-α), chemokines including macrophage inflammatory protein-1β and monocyte chemotactic protein-1, prostaglandins (cox 2), growth factors such as macrophage colony stimulating factor, and complement components (C1q, and C1 to C9) [19, 20]. Additional neurotoxic compounds produced by activated microglia include superoxide, hydrogen peroxide, and nitrous oxide [156]. Oxidative stress (lipid oxidation, protein oxidation, DNA oxidation, and glycol-oxidation) contributes to neurodegeneration in AD [159]. Associated pathological processes include endoplasmic reticulum stress associated with change in endoplasmic reticulum calcium homeostasis [160, 161], release of free electrons from dysfunctional mitochondria [162], and formation of reactive oxygen species [163].

Infection with either HSV-1 [10] or *C. pneumoniae* [12] induces $A\beta_{42}$ deposits and plaques, and *H. pylori* filtrate [148] results in elevated levels of $A\beta_{42}$ in animal brain models. *In vitro* infection by HSV-1 [10], *B. burgdorferi* [13], and *H. pylori* filtrate [148] induces Aβ deposition in mammalian neuronal or neuronal/glial cell models.

Aβ has been shown *in vitro* to be an anti-microbial peptide against eight specific microorganisms, including *Escherichia coli, Streptococcus pneumonia*, and *Candida albicans*. AD whole brain homogenates have significantly higher antimicrobial activity compared to age matched non-AD control samples [164]. $A\beta_{42}$ has shown anti-microbial peptide properties by attenuating HSV-1–induced miRNA-146a levels in human neuronal-glial cell cultures and significantly reducing pathological HSV-1 effects on cultured brain cells [165]. Aβ production may be part of the CNS immune response to infection with eventual harmful effects to neurons due to overproduction of Aβ [166].

Evidence supporting the role of the adaptive immune system in AD

Lynch has proposed that BBB permeability, which is increased in AD, together with the creation of a chemotactic gradient, leads to infiltration of IFN-γ-producing T cells into the AD brain [167]. T cell production of IFN-γ induces classical microglia activation, which leads to inflammatory cytokine and chemokine production. This in turn results in increased AβPP processing, Aβ accumulation, further BBB permeability, and infiltration of more T cells, leading to a continuous cycle of neurodegeneration (Fig. 6) [167].

Resident cells in the brain produce only limited IFN-γ [167]. Under normal conditions, T cell entry into the CNS is limited and thought to be related to T cell immuno-surveillance [168, 169]. Significant infiltration of immune cells does occur in neuroinflammatory conditions [170] and T cells have been localized in the brains of AD patients [171–178]. Breakdown of the BBB (see BBB section below), increased expression of T cell attractant chemokines such as interferon-γ-inducible protein 10 (IP-10), and corresponding chemokine receptors such as CXCR3 on neuronal cells have been found in AD brains [179] and may contribute to infiltration of T cells into the AD brain [167].

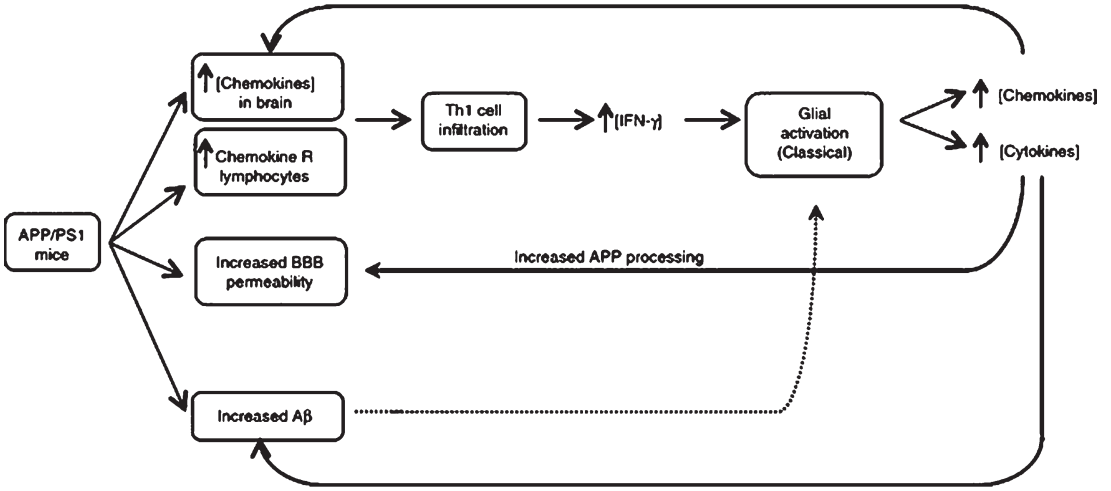

Fig. 6. The Lynch Hypothesis: T-cell lymphocytes infiltrate the brain and secrete IFN-γ which induces microglia activation and contributes to neurodegeneration in AD. Proposed sequence of events leading to amyloid pathology and microglial activation in AD. T lymphocytes cells activated peripherally cross the BBB and secrete IFN-γ and other cytokines, interact with microglia, and influence the neurodegenerative processes involved in AD. Figure from Lynch MA (2014) The impact of neuroimmune changes on development of amyloid pathology; relevance to Alzheimer's disease. *Immunology* 141, 292-301 [167]. Copyright 2014. Reprinted with permission from John Wiley and Sons, Inc. and Marina Lynch.

T cells can interact with microglia to modulate their function, as demonstrated by *in vitro* co-culture experiments [167]. Resting microglia cells from BALB/c mice developed features of antigen presenting cells, including strongly upregulated surface expression of MHCII, CD40, and CD54 when co-cultured with type 1 T helper cells (Th1) [180]. Conversely, mouse microglia induce Th1 cells to release IFN-γ [180]. Supernatants produced by allo-antigen and myelin basic protein-specific human pro-inflammatory Th1 T-cell lines augmented expression of cell surface molecules MHC class II, CD80, CD86, CD40, and CD54, enhanced the functional antigen-presenting cell capacity in a mixed lymphocyte reaction, and increased cytokine/chemokine secretion (TNF-α, IL-6, and CXCL10/IP-10) by CNS-derived human microglia [181]. Co-culture of Aβ-specific Th1 or Th17 cells and microglia induced pro-inflammatory cytokine production (IL1-β, TNF-α, and IL-6) and antigen presenting cell capacity of microglia in a murine model [182]. IFN-γ activates murine microglial cells and results in microglial production of TNF-α and inducible nitric oxide synthase (i-NOS) *in vitro* [183].

Browne and colleagues investigated the role of Aβ-specific T cells on Aβ accumulation in transgenic mice that overexpress AβPP and presenilin 1 (AβPP/PS1 mouse model), and found significant infiltration of T cells in these brains. Aβ-specific CD4+ T cells were generated by immunization with Aβ and a TLR agonist and polarized *in vitro* to Th1-, Th2-, or IL-17-producing CD4+ T cells. A proportion of these T cells secreted IFN-γ or IL-17. These Aβ-specific T cells were then adoptively transferred to AβPP/PS1 mice at 6 to 7 months of age. At 5 weeks, Th1 cells, but not Th2 or IL-17-producing CD4+ T cells had infiltrated into these brains. Additionally, there was increased microglial activation and CNS Aβ deposition. All of these findings were associated with impaired cognitive function. Treatment of the AβPP/PS1 mice with an anti-IFN-γ antibody attenuated the Th1 cell effects. The authors suggest that the release of IFN-γ from infiltrating Th1 cells significantly accelerates markers of diseases in an animal model of AD [184]. Murphy *et al*. demonstrated that murine Th17 and CD4+ lymphocytes, which produce IL-17 and IFN-γ, induce microglial production of IL-1β, IL-6, and TNF-α in experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis [185]. The combination of IFN-γ and TNF-α has been shown to induce the production of Aβ peptides and inhibit the secretion of soluble AβPP by human neuronal and extraneuronal cells *in vitro* [186].

These findings lend support to the hypothesis that T lymphocytes activated peripherally may cross the BBB and secrete IFN-γ and other cytokines, interact with microglia, and influence the neurodegenera-

tive processes involved in AD. Thus, T cells may be an important link between the systemic adaptive immune system and the innate immune system in the AD brain.

Infection-induced acute or chronic inflammation exacerbates tau pathology in vivo

Sy *et al*. demonstrated that acute inflammation induced by viral infection or chronic inflammation induced by bacterial LPS resulted in AD-like pathology in animal brains using the triple transgenic AD mouse model (3xTg-AD). Aged 3xTg-AD and non-Tg mice infected with a single dose of mouse hepatitis virus (MHV) by injection into the hippocampus developed similar acute neuroinflammatory responses with increased infiltration into the brain by macrophages, CD4+ T cells, CD8+ T cells, and activation of microglia. After viral clearance at 2 and 4 weeks post-infection, MHV-infected 3xTg-AD mice showed a marked increase in phosphorylated tau protein levels in the brain. In addition, increased activation of GSK-3β, one of the enzymes that phosphorylates tau protein, was found. This effect was not seen in MHV infected non-Tg mice. Sustained brain inflammation was induced in 3xTg-AD aged mice by intraperitoneal injection of lipopolysaccharide, which is an outer membrane Gram-negative bacterial endotoxin that simulates bacterial infection. Injection of LPS twice weekly for 6 weeks resulted in significant elevation of phosphorylated tau protein levels, increased GSK-3β activity, and cognitive decline compared with saline injected 3xTg-AD aged control mice. Based on these findings, the authors suggest that certain microbial infections may act as comorbid factors in the pathogenesis of AD by inducing inflammation, increasing levels of phosphorylated tau proteins, and exacerbating cognitive decline [187].

Infectious burden is associated with systemic inflammation and serum Aβ levels in AD

Bu and co-workers studied IB, serum cytokine, and Aβ levels, and cognition in 128 AD patients and 135 healthy controls. IB consisted of serum antibody levels to CMV, HSV-1, *B. burgdorferi*, *C. pneumoniae*, and *H. pylori*. Total IB, bacterial burden, and viral burden were independently associated with AD after adjusting for age, gender, education, *APOE* genotype, and other comorbidities. They found a significant association between AD and an increasing number of pathogens to which an individual had been exposed, with an OR of 3.988 in patients seropositive for 4-5 pathogens compared with those seropositive for 0–2 pathogens. Furthermore, higher IB was associated with higher serum Aβ levels in both AD and healthy controls with seropositivity to 3 or more pathogens. There were significantly higher serum levels of IFN-γ, TNF-a, IL-1β, and IL-6 in participants seropositive for 4-5 pathogens than those seropositive for 0–2 pathogens when including all cases. AD patients seropositive to 4-5 pathogens exhibited significantly higher levels of IFN-γ, TNF-α, and IL-6 when compared with AD patients seropositive to 0–2 pathogens. The authors suggest that higher levels of IB may promote the development of AD by infection-induced inflammation and elevated Aβ levels [188].

APOLIPOPROTEIN E OVERVIEW

Apolipoprotein E (ApoE) is a 299 amino acid protein component of lipoproteins. The primary metabolic role for ApoE is to shuttle and distribute lipids from one tissue or cell type to another and to regulate lipid metabolism [189]. Various ApoE isoforms appear to play a role in disease susceptibility and outcome of certain infections, with evidence also supporting involvement in immune regulation [189]. The liver synthesizes the majority of ApoE; however, 20–40% of ApoE is produced by extra-hepatic cells including glial cells [190] and neurons [191].

ApoE associates with lipoproteins including VLDL, LDL, and HDL during systemic transport of triglycerides and cholesterol and is a primary carrier of lipids across the BBB into the brain [190]. When carrying lipids, ApoE binds to members of the low density lipoprotein receptor (LDLR) family [191]. A secondary proposed ApoE binding site involves the heparan sulphate proteoglycan (HSPG)/LDL-C receptor-related protein pathway [192].

The human *APOE* gene is located on chromosome 19 as a single gene locus with three major allele isoforms designated ε4, ε3, and ε2 [189]. *APOE* allele frequencies vary between ethnicities, with the *APOE*-ε4 allele variant occurring at a frequency of 5–30%, the *APOE*-ε3 allele variant at 50–90%, and the *APOE*-ε2 allele variant at 0–15% [190]. The corresponding products of these alleles are the ApoE isoforms named ApoE4, ApoE3, and ApoE2. The 6 resultant ApoE phenotypes include 3 homozygous phenotypes (E4/4, E3/3, and E2/2) and 3 heterozygous phenotypes (E4/3, E4/2, and E3/2) [190].

The APOE-ε4 allele impacts outcomes in certain infections

As discussed above, Itzhaki demonstrated increased risk of AD by a factor or 12 in *APOE-ε4* carriers who have HSV-1 in the brain [49]. Patients with the *APOE-ε4* allele had a higher rate of oral herpetic lesions compared to non-*APOE-ε4* allele carriers with a relative risk of 4.64 [193]. Transgenic APOE4 mice infected with HSV-1 demonstrate higher CNS viral loads compared with APOE3 [51]. HSV-1 binds to HSPG located on the target cell membrane to facilitate entry into the cell [194]. Itzhaki *et al*. suggest that ApoE4 may compete with HSV-1 for binding to cell surface HSPG receptors, and that ApoE4 is less competitive than ApoE3 or ApoE2, allowing more virus particles to gain entry into the cell [191].

C. pneumoniae elementary bodies bind to human astrocytoma cells possessing the *APOE-ε4* allele at levels 3-fold higher than non-*APOE-ε4* allele bearing cells. A separate line of astrocytoma cells transfected with plasmids expressing the ε4 coding sequence had 3-fold more *C. pneumoniae* elementary body attachment than astrocytoma cells encoding ApoE3. These findings demonstrate that expression of ApoE4 enhances attachment of *C. pneumoniae* elementary bodies to target host cells, which may enhance infectivity [195].

APOE-ε4 allele carriage is associated with a reduced risk of acquiring chronic hepatitis C virus (HCV) [190, 196]. Carriage is also protective against severe liver disease caused by HCV [197]. The exact mechanism for this protective effect has yet to be elucidated; however, HCV associates with serum lipoproteins including apoE to enter cells via the LDLR [198]. The expression of LDLR on the cell surface of hepatocytes is inversely related to the concentration of LDL in serum [199]. One hypothesis suggests that a higher serum LDL concentration in *APOE-ε4* carriers leads to a lower LDLR expression, which potentially decreases virus entry and spread between hepatocytes [197].

Corder and colleagues phenotyped sera from HIV patients for ApoE and found that patients who possessed a single copy of the ApoE4 isoform had significantly higher rates of dementia and peripheral neuropathy compared to those who were ApoE4 negative [200]. Burt *et al*. demonstrated that HIV positive patients with the *APOE-ε4/ε4* genotype have both accelerated disease progression and progression to death compared to *APOE-ε3/APOE-ε3* carriers. An association between *APOE-ε4/ε4* genotype and HIV-associated dementia was not confirmed in this study. However, using an *in vitro* cell model with specialized HeLa cells, the authors found that the HIV infection rate was significantly higher in the presence of ApoE4 compared with ApoE3 [201].

The target cells of HIV include CD4+ T-cells, macrophages, and microglia cells. HIV initiates entry into the cell by attaching to the HSPG receptor on the target cell membrane [190]. HIV envelope glycoproteins then bind to the CD4 receptor. Fusion to the cell membrane requires cholesterol in HIV particles and lipid rafts, which are cell membrane micro domains enriched in certain lipids, cholesterol and proteins [190]. The mechanism by which ApoE isoforms impact HIV disease outcomes is not known. Research has focused on the differential effects of ApoE isoforms on HIV particle binding activity and uptake involving the LDL-R and the HSPG receptors [190]. One proposed mechanism is that ApoE4 may be less competitive compared with HIV at the target cell HSPG receptor than ApoE3 or ApoE2 resulting in increased HIV cell entry [190]. An additional hypothesis is that ApoE4 on the HIV viral envelope promotes HIV binding activity at the low-density lipoprotein receptor of the target cell [190].

APOE-ε4 allele is associated with enhanced human innate immune responses

Gale *et al*. demonstrated that carriage of the *APOE-ε4* allele is associated with enhanced *in vivo* innate immune responses in human subjects [202]. Whole blood from healthy genotyped *APOE-ε3/APOE-ε4* volunteers exposed *ex vivo* to TLR2, TLR4, or TLR5 ligands induced significantly higher levels of TNF-α than blood from *APOE-ε3/APOE-ε3* carriers. Blood from *APOE-ε3/APOE-ε4* carriers also induced significantly higher levels of IL1-β, IL-6, IFN-γ, and additional cytokines and chemokines after exposure to TLR2 or TLR4 when compared with blood from *APOEε3/APOEε3* carriers. No difference was seen between the two ApoE phenotypes regarding production of anti-inflammatory compounds IL-4 and IL-1 receptor antagonist. Thus, ApoE4 is associated with a broad pro-inflammatory response to TLR cascades. Human *APOE-ε3/APOE-ε4* subjects exposed to an intravenous LPS challenge demonstrated enhanced immune responses with significantly higher plasma levels of TNF-α and greater sustained body temperatures compared with *APOE-ε3/APOE-ε3* subjects [202].

Differences in monocyte lipid rafts have been found in *APOE-ε3/APOE-ε4* human peripheral blood monocytes compared with *APOE-ε3/APOE-ε3* monocytes [202]. Lipid rafts within the cell membrane organize cellular signaling events [203]. Several TLR cascades are initiated in lipid rafts, which and are enhanced by cholesterol loading [202]. ApoE4 is less efficient at inducing cholesterol efflux and removing cholesterol in mouse macrophages [204]. Gale *et al*. used a fluoro-tagged cholera toxin B subunit lipid raft probe to study *in vitro* CD14 monocytes. Augmented lipid raft assembly in *APOE-ε3/APOE-ε4* monocytes compared to *APOE-ε3/APOE-ε3* monocytes was demonstrated. The authors suggest that ApoE4 may enhance cholesterol loading in monocyte lipid raft structures due to decrease in cholesterol efflux, which in turn enhances TLR responses with pro-inflammatory consequences [202].

The *APOE-ε4* genotype with resultant ApoE4 phenotype appears to influence disease outcome for several infections and elevate the pro-inflammatory innate immune response. Evidence supporting ApoE4 associated enhanced infectivity and brain infiltration would explain the increased risk of AD in patients who are co-factor positive for HSV-1 in the brain and the *APOE-ε4* allele [50]. Increased binding of *C. pneumoniae* elementary bodies to *APOE-ε4* target host cells suggests increased infectivity as a mechanism to explain elevated numbers of *C. pneumoniae* infected cells in *APOE-ε4* allele carrier AD brains [123]. An increased ApoE4 associated pro-inflammatory response may contribute to AD pathogenesis by both increasing BBB permeability and elevating levels of pro-inflammatory cytokines.

THE BLOOD-BRAIN BARRIER AND THE AD PATHOGEN HYPOTHESIS: BBB IMPAIRMENT IN AD

Breakdown of the BBB with increased permeability is involved in the pathophysiology of AD [205, 206]. Postmortem studies have shown BBB damage in AD with accumulation of blood-derived proteins including albumin, fibrinogen, thrombin, and immunoglobulins in the hippocampus and cortex [207]. BBB breakdown appears to be an early event in the aging human brain that begins in the hippocampus and may contribute to cognitive impairment [207]. Montagne and collaborators used an advanced dynamic contrast enhanced MRI protocol with high spatial and temporal resolutions to quantify regional BBB permeability in the living human brain. In doing so, they demonstrated an age-dependent BBB breakdown in the hippocampus, an area of the brain affected early in AD. The BBB breakdown in the hippocampus and its CA1 and dentate gyrus subdivisions worsened with MCI that correlated with injury to BBB-associated pericytes, as shown by the CSF analysis [207]. Evidence suggests that pro-inflammatory cytokines, Aβ, and APOE4 genetics are contributing factors in the breakdown of the BBB [212–214], with data indicating pathogen association and interactions with each of these factors as previously discussed.

Composition of the blood-brain barrier

The BBB is located at the level of the cerebral microvasculature and serves as the largest interface for blood-brain exchange [208]. The BBB forms a physical, enzymatic, and transport barrier between the vasculature and the brain parenchyma [209]. Brain microvascular endothelial cells (BMECs) line cerebral blood vessels to form a barrier on the endothelial side of the vessel. Pericytes and astrocyte end feet form a continuous barrier in association with the basement membrane on the adluminal vessel surface [210]. BMECs form intercellular contacts via two types of junctions known as adherens junctions (AJs) and tight junctions (TJs). TJs in BMECs are composed of the membrane proteins occludin, claudins, junctional adhesion molecules, and cell-selective adhesion molecules, which are linked to the actin cytoskeleton by cytoplasmic proteins ZO-1, ZO-2, ZO-3, and cingulin [210]. AJs contain cadherins bound to actin microfilaments by a submembranous zone of catenins [211]. These junctions promote high transendothelial electrical resistance that restricts paracellular permeability [210]. This in turn blocks the transport of a wide range of molecules and regulates the passage of ions, macromolecules, and polar molecules from the systemic circulation [210]. Endothelial cells of the BBB lack fenestrations and have a reduced number of pinocytotic vesicles, which restricts transcellular flux [208]. However, some molecules and solutes are transported across the BBB by various mechanisms including transporters, receptor and/or adsorption-mediated transcytosis, and passive diffusion [208].

The blood-brain barrier: Pathogens and neuroinflammation

Certain pathogens may cross the BBB either transcellularly, paracellularly, or by means of infected phagocytes by a Trojan horse mechanism [215]. *N. meningitidis* exemplifies a bacterium which crosses the BBB transcellularly [215]. Evidence suggests that *B. burgdorferi* may cross the BBB paracellularly [215], whereas *C. pneumoniae* may cross by way of the "Trojgan horse" mechanism, intracellulary within monocytes or macrophages [108, 109]. Other pathogens including HSV-1 cross the BBB via the olfactory nerve and/or trigeminal nerve [5, 208]. In addition to pathogens that cross the BBB directly, blood-born cytokines including IL-1α, IL1-β, IL-6, TNF-α, and others can cross BBB via transport systems and act directly on brain tissue as demonstrated in animal models [209, 216].

CNS infections and other diseases associated with elevated pro-inflammatory cytokine levels increase the permeability of the BBB [214]. Cytokines such as IL-1 act on brain endothelial cells to increase the expression of endothelial adhesion molecules and chemokines, including CC chemokine ligand-2 (CCL2), selectins, and intracellular adhesion molecule-1 (ICAM-1), which contribute to BBB permeability [217]. Pro-inflammatory cytokines also increase expression of matrix metalloproteinases (MMPs), which degrade extracellular matrix components in the endothelial basement membrane and tight junctions, resulting in increased BBB permeability [218, 219]. Labus *et al*. found that IL-1β induced an inflammatory response and breakdown of the endothelial layer in an *in vitro* BBB model. IL-1β induced endothelial cells expression of adhesion molecule ICAM-1, IL-6, IL-8, and TNF- α. IL-1β also reduced the expression of tight junction protein ZO-1. Increases in both paracellular permeability and leukocytes crossing the cell layer of the BBB model were demonstrated [214].

Aβ and the blood-brain barrier

Evidence indicates that Aβ and BBB disruption interact to mutually promote their effects on AD pathogenesis [213]. Accumulation of Aβ in the brain may contribute to the breakdown of the BBB. Conversely, dysfunction of the BBB may result in Aβ accumulation due to BBB leakage or decreased clearance [213]. P-glycoprotein, an efflux pump highly expressed on the luminal surface of brain capillary endothelial cells of the BBB, has been shown to transport Aβ from the brain to the blood [220, 221]. Decreased function of this transporter has been reported in AD brains [221]. $A\beta_{42}$ is able to modify the expression of TJs and alter the functionality of an epithelial BBB *in vitro* model [222]. In addition, Aβ accumulation has been shown to cause BBB dysfunction by inducing endothelial cell toxicity both *in vitro* and *in vivo* in animal studies, human studies, and human postmortem studies [223]. Soluble Aβ also stimulates BBB endothelial cells to increase monocyte adhesion, which has been hypothesized to increase monocyte permeability across the BBB leading to monocyte transmigration into the brain parenchyma [223].

APOE-ε4 and the blood-brain barrier

Animal models support a role for genetic susceptibility in the breakdown of the BBB. Using *APOE* transgenic mice, Bell *et al*. found that expression of ApoE4 and lack of murine ApoE lead to BBB breakdown by activating a pro-inflammatory cyclophilin A (CypA)-nuclear factor-κB-MMP-9 pathway in pericytes through a lipoprotein receptor. This was followed by neuronal uptake of neurotoxic proteins and reductions in microvascular and cerebral blood flow. Breakdown in the BBB preceded neuronal dysfunction and the initiation of neurodegenerative changes [224].

In a postmortem study, Halliday and colleagues demonstrated accelerated pericyte degeneration in AD *APOE-ε*4 carriers > AD *APOE-ε*3 carriers > non-AD controls, which correlated with the magnitude of BBB breakdown as measured by permeability to immunoglobulin G and fibrin. Accumulation of CypA and MMP-9 in pericytes and endothelial cells was greater in AD subjects who were *APOE-ε*4 carriers than those who were *APOE-ε*3 carriers. Levels of the ApoE lipoprotein receptor, low-density lipoprotein receptor-related protein-1 (LRP1), were reduced in AD *APOE-ε*4 and *APOE-ε*3 carriers. This data suggests that possession of *APOE-ε*4 leads to accelerated pericyte loss and enhanced activation of LRP1-dependent CypA-MMP-9 BBB-degrading pathway in pericytes and endothelial cells. These processes mediate BBB damage, which is more severe in AD *APOE-ε*4 carriers than AD *APOE-ε*3 carriers [212].

POTENTIAL ANTIMICROBIAL TREATMENTS FOR AD CLINICAL TRIALS

Treatment of HSV-1: Acyclovir and valacyclovir

Acyclovir decreases HSV-1-induced Aβ accumulation in cultured neuroblastoma cells [166]. Aβ in cell cultures was reduced by 70% at a 200 μM concentration of acyclovir. In addition, acyclovir inhibits HSV-1-induced abnormal tau phosphorylation *in vitro*. Abnormal tau phosphorylation was reduced nearly 100% at a 200 μM concentration of acyclovir (Fig. 7) [166]. Acyclovir decreased Aβ by reducing cellular viral spreading and decreased tau phosphorylation by interfering with viral replication [166]. Penciclovir and foscarnet, which also inhibit viral DNA replication, were shown to reduce phosphorylated tau and Aβ, with foscarnet being less effective than acyclovir and penciclovir [166].

Acyclovir is a nucleoside analogue that interferes with HSV-1 replication and reactivation. Viral thymidine kinase is required to convert acyclovir into acyclo-guanosine monophosphate. Subsequently, the monophosphate form is converted to the active triphosphate form by cellular kinases. As a substrate, acyclo-guanosine triphosphate is incorporated into viral DNA, resulting in premature chain termination. Acyclovir is able to cross the BBB [225]. The drug would directly target a potential cause of AD, act on infected cells only, and would not affect the normal metabolism of infected neurons [166].

Acyclovir is FDA approved and widely used for the treatment of HSV infections. The side effect profile is mild; however it would be necessary to monitor renal function [225]. Rarely, treatment is complicated by reversible neuropsychiatric symptoms occurring more frequently in patients with pre-existing renal impairment [225].

Valacylovir is the biodrug of acyclovir. Following oral administration, valacyclovir is rapidly hydrolyzed to acyclovir via first-pass intestinal and hepatic metabolism [225]. Valacyclovir has better oral absorption than acyclovir and is able to cross the BBB as acyclovir following hydrolysis [226]. Oral valacyclovir has been used to successfully treat herpes simplex encephalitis [227]. In a multiple sclerosis trial evaluating HHV-6, valacyclovir at a dose of 3 grams per day for 2 years was shown to be safe with no patient discontinuation due to side effects or toxicity [228]. Both acyclovir and valacyclovir have been found safe during long-term use in patients being treated for HSV suppression [229]. Furthermore, resistance rates are low (<0.5%) among immunocompetent patients [229].

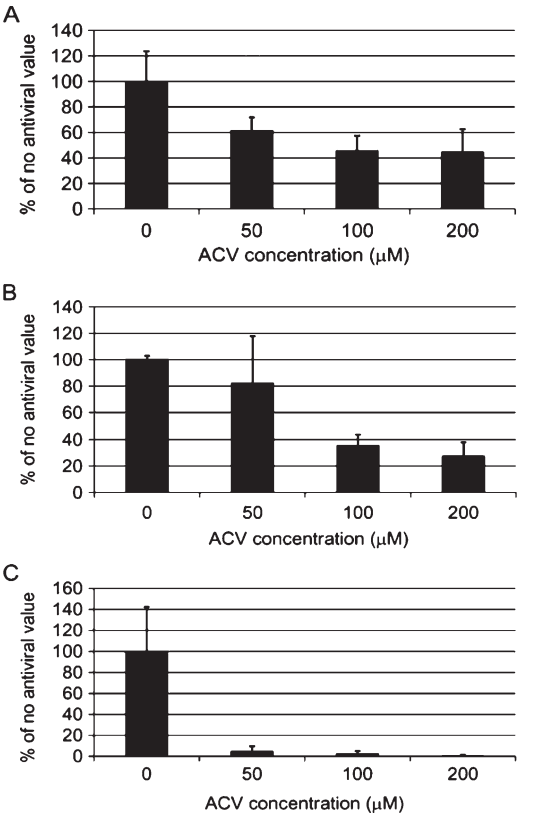

Fig. 7. Quantification of HSV-1 proteins (A), amyloid-β (B), and abnormal tau phosphorylation (C) in HSV-1-infected cells after acyclovir treatment. HSV-1 infected vero cell cultures treated with 0 μM, 50 μM, 100 μM, or 200 μM acyclovir (ACV). ACV significantly inhibited replication of HSV-1 as shown by a decrease in HSV-1 proteins (A). Aβ in cell cultures was reduced by 70% at a 200 μM concentration of acyclovir (B). Abnormal tau phosphorylation was reduced nearly 100% at a 200 μM concentration of acyclovir (C). $p < 0.0001$ compared to controls at all ACV concentrations for A and C and at 100 μM and 200 μM ACV concentrations for B. Graph from Wozniak MA *et al.* (2011) Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with Herpes simplex virus type 1. *PLoS One* **6**, e25152 [166]. Copyright 2011. Reprinted under the terms of the Creative Commons Attribution License, (http://creativecommons.org/licenses/by/2.0) and permission from Ruth Itzhaki.

Prasad *et al*. conducted a clinical trial involving 24 HSV-1 IgG seropositive schizophrenia patients who were treated with valacyclovir 1.5 grams twice daily for 18 weeks. The treatment group demonstrated significantly improved cognition, specifically

in verbal memory, working memory, and visual object learning, compared with the HSV-1 IgG seronegative schizophrenia control group. Both treatment and control groups were treated with anti-psychotic medication. While psychotic symptoms did not improve with valacyclovir, the study did show that valacyclovir improved cognition in a select group of HSV-1 exposed neuropsychiatric patients [230].

Intravenous immunoglobulin (IVIG)

Natural antibodies to Aβ in human IVIG promote microglial recognition and removal of natively formed human Aβ deposits *ex vivo* in an AβPP/PS1 mouse model of AD [231]. IVIG administered peripherally *in vivo* crossed the mouse BBB, reaching highest concentrations in the hippocampus. The antibodies bound selectively to Aβ deposits and co-localized with microglia [231]. Preclinical and small clinical studies treating early stage AD patients with IVIG showed decreases in CSF Aβ levels and increases in serum Aβ levels compared to baseline, with reductions in cognitive decline compared to controls [232, 233]. However, the Gamma Globulin Alzheimer's Partnership (GAP) study, a large double blind IVIG clinical trial treating mild to moderate AD patients, did not meet primary outcome objectives regarding cognitive function and activities of daily living. Biomarker studies did indicate dose dependent increases in plasma and CSF immunoglobulins and decreases in plasma $A\beta_{42}$ levels [234].

IVIG has antiviral activity against HSV-1 and can neutralize extracellular virus [235]. Additionally, IVIG in conjunction with lymphocytes is able to destroy cells infected with HSV-1 [235–237]. IVIG (Privigen) treated HSV-1 infected Vero cells demonstrated statistically significant reductions of Aβ, phosphorylated tau, and HSV-1 proteins [238]. Privagen appeared to prevent viral entry into cells and act synergistically with acyclovir, leading the authors to suggest that the combination of IVIG and acyclovir may be beneficial in the treatment of AD [32, 238].

HSV-1 vaccine

Lin and coworkers showed that a vaccine of mixed HSV-1 glycoproteins had a protective effect against HSV-1 infection of mouse brain. The vaccine significantly reduced HSV-1 latency in the CNS of mice that had been infected peripherally with HSV-1 [239]. Although not yet developed, a human vaccine for HSV-1 administered to prevent HSV-1 infiltration into the brain might prevent some cases of AD [239].

Treatment of CMV, EBV, and HHV-6

Anti-viral therapy for asymptomatic CMV infection in immunocompetent patients is not feasible because of toxicity, limited number of approved drugs, and the potential for drug resistance [82]. Thus, there is no available suppressive therapy for CMV comparable to the usage of valacyclovir or acyclovir for HSV suppression. Ganciclovir (GCV) and valganciclovir, the oral prodrug of GCV, are nucleoside analogues. GCV is phosphorylated to its active form in CMV-infected cells. These medications are currently the most commonly prescribed drugs for the prevention and treatment of CMV in immunocompromised patients [240]. Although limited, current evidence suggests that targeted antiviral therapy with GCV or valganciclovir is appropriate for severe CMV disease in immunocompetent adults [241]. There are no FDA approved medications for the treatment of EBV, and anti-viral therapy is generally ineffective for this virus [242]. No therapies are approved for the treatment of HHV-6 currently; however, small studies and individual case reports have reported intermittent success with drugs such as cidofovir, GCV, and foscarnet [243]. To date there are no FDA approved vaccines for CMV, EBV, or HHV-6 [240].

Treatment of Chlamydophila pneumoniae

Acute *C. pneumoniae* infections are susceptible to antibiotics that interfere with DNA and protein synthesis, including tetracyclines, macrolides, quinolones, and rifamycins [110]. All of these antibiotics cross the BBB except for the macrolides [244]. *C. pneumonia*e becomes spontaneously persistent following infection of monocytes [112]. *C. pneumonia*e within infected lymphocytes *in vitro* demonstrated resistance to single antibiotics including minocycline and tosufloxacin and did not show uniform susceptibility within infected monocytes [245]. Nine months of combination doxycycline and rifampin were used in a successful clinical trial as treatment for chronic *Chlamydia*-induced reactive arthritis. This study suggests that persistent chlamydia infection responds poorly to single antibiotic therapy but appears to be susceptible to combination antibiotic regimens [246].

Tetracyclines possess both immunomodulatory and antiapoptotic properties [247] and have been

shown to be anti-inflammatory and neuroprotective in various models of neurodegenerative disease [248]. Tetracycline and doxycycline exhibit anti-amyloidogenic activity *in vitro* by inhibiting the self-aggregation capacity of $A\beta_{42}$ and disassembling pre-formed $A\beta_{42}$ fibrils [249]. The semi-synthetic, second generation tetracycline analog minocycline inhibited increases in p-eIF2α and reduced neuronal cell death in $A\beta_{42}$ peptide treated nerve growth factor-differentiated rat pheochromocytoma (PC12) cells *in vitro* [161]. Minocycline also reduced neuronal cell death and attenuated deficits in learning and memory after $A\beta_{42}$ infusion in a rat model of AD [161].

Rifampin crosses the BBB [244, 250] and attenuates all chlamydial gene transcription [246]. Rifampin-induced upregulation of LRP1 and p-glycoprotein at the BBB enhances Aβ clearance from the mouse brain [251].

In a clinical trial, Loeb *et al*. treated probable AD patients diagnosed with mild to moderate dementia with doxycycline and rifampin versus placebo for 3 months. There was significantly less decline in cognition as measured by the Standardized Alzheimer's Disease Assessment Scale cognitive subscale at 6 months in the antibiotic treatment group compared to the placebo group [252]. In a clinical trial by Molloy and colleagues, mild to moderate AD patients were treated for 12 months with doxycycline and rifampin. These drugs, given both alone or in combination, had no effect on cognition or function [253]. Pre-clinical AD patients and biomarker endpoints were not assessed in this study. In addition, these medications have not been tested in combination with valacyclovir.

Treatment of spirochetes

The CNS infection Lyme neuroborreliosis caused by the spirochete *B. burgdorferi* has been successfully treated in clinical trials with 10–14 days of intravenous ceftriaxone or oral doxycycline [254, 255]. In one trial, 79% of the ceftriaxone-treated patients and 72% of the doxycycline-treated patients had completely recovered by 6 month follow-up. Ceftriaxone and oral doxycycline used as single agents were found to be effective, safe, and convenient for treatment of Lyme neuroborreliosis [254]. Abramson *et al*. studied the bactericidal activity of antimicrobial agents *in vitro* for 17 strains of treponemes. Most treponemes, including human oral spirochetes such as *T. dentolytica*, were sensitive to tetracycline, doxycycline, and cephalothin [256].

Treatment of periodontal pathogens

Improved oral hygiene and meticulous dental care may be beneficial for patients with dementia. Kikutani *et al*. studied the effects of oral care in a group of nursing home vascular dementia and AD patients. The oral care group had a high level of dental care, as nurses and caregivers cleaned the patients' teeth and mouth with a toothbrush for approximately 5 minutes after each meal for one year. The oral care group had significantly less decline in MMSE scores compared to the non-oral care group at six months and one year [257].

Systemic antibiotics are widely used in the treatment of periodontal infections; however, clear guidelines for the use of systemic antibiotics to treat periodontitis in general clinical practice are not yet available [258, 259]. Adjunctive antibiotic treatment in moderate periodontal disease for 14 days with either amoxicillin or metronidazole in addition to traditional scaling and root planing has been shown to markedly reduce bacterial counts for pathogenic species including *B. forsythus*, *P. gingivalis*, and *T. denticola*, which remained lower than baseline at one year post-treatment [259]. Several clinical trials treating patients with mild to moderate periodontal disease have shown microbiological and/or clinical benefits by using azithromycin or metronidazole combined with scaling and root planing or pocket reduction surgery when compared to scaling and root planing alone [260–262], or surgery alone [263]. Sub-antimicrobial dose doxycycline (20 mg doxycycline twice daily) has also been used successfully to treat periodontitis [247]. Feres *et al*. extensively reviewed randomized clinical trials performed over the last decade involving antibiotic treatment of periodontitis where advanced microbial diagnostic testing had been performed. Patients treated with adjunctive antibiotic therapy had improved microbiological and clinical outcomes [258]. The authors recommended that patients with advanced or very advanced periodontitis should be treated with the adjunctive use of metronidazole or combination metronidazole plus amoxicillin for 14 days in addition to traditional scaling and root planing [258]. Antibiotic treatment does not appear to create lasting changes in the percentage of resistant isolates or sites harboring resistant species [260, 264].

Treatment of Helicobacter pylori

Treatment of *H. pylori* involves triple or quadruple regimens using a proton pump inhibitor such as omeprazole plus various combinations of amoxicillin, Clarithromycin, metronidazole, and tetracycline taken orally for 7 to 14 days. Sequential, concomitant, or hybrid regimens are chosen depending on resistance rates and other clinical factors [265, 266]. Clarithromycin and metronidazole resistance has been increasing in certain populations. Levaquin triple based and bismuth quadruple based regimens have been used successfully to treat resistant *H. pylori*. Patient-specific therapy is based on factors such as cost, allergy history, and known or suspected patterns of resistance [266]. Treatment success rates with current regimens are variable, ranging from 50% to over 95% in clinical trials depending on factors such as length of treatment, rates of antibiotic resistance and patient specific cytochrome P450 2C19 genotype [265].

Kountouras *et al*. evaluated AD patients with gastric biopsy-proven *H. pylori* infection. Patients were treated with combination omeprazole, clarithromycin, and amoxicillin triple based therapy to eradicate *H. pylori* from the gastric mucosa. At the 2-year clinical endpoint, cognitive (MMSE and Cambridge Cognitive Test) and functional (Functional Rating Scale for Symptoms of Dementia) parameters significantly improved in the subgroup of AD patients with successful *H. pylori* eradication, but not in the subgroup of AD patients in which *H. pylori* eradication was not achieved [152]. In another clinical trial, successful treatment and eradication of *H. pylori* in a group of probable AD patients was associated with a significantly lower 5-year mortality risk than a control group of probable AD patients who did not achieve eradication of the bacterium [267].

CONCLUSION

Evidence supports the hypothesis that pathogens interact with susceptibility genes and are causative in AD. Pathogen induced neurodegeneration occurs by both direct effects on brain cells and indirect inflammatory and oxidative effects. HSV-1, *C. pneumoniae*, and spirochetes are able to infect the brain and induce formation of Aβ and hyperphosphylated tau proteins. Chronic CNS infections lead to glial cell activation, resulting in neuroinflammation and neuronal apoptosis. Peripheral infections including CMV, periodontal pathogens, and *H. pylori* induce systemic inflammation with elevated levels of pro-inflammatory molecules. Pathogen induced pro-inflammatory cytokines are transported across the BBB into the brain where they induce CNS inflammation and contribute to AD pathology in genetically susceptible individuals. In addition, pathogen induced pro-inflammatory cytokines and Aβ interact with genetic susceptibility factors to damage the BBB, resulting in increased BBB permeability. Chronic CMV infection with reactivation in peripheral blood or organ systems induces systemic activation of IFN-γ-producing T cells, which may enter the brain and contribute to the pathogenesis of AD. CMV also plays a role in immunosenescence, which damages the immune system and is associated with the reactivation of other *Herpesviridae* such as HSV-1. Thus, in addition to direct pathogen effects, AD pathogenesis results from interactions involving both the innate and adaptive immune responses to both CNS and peripheral systemic pathogens.

As proposed, the AD pathogen hypothesis would explain the multiple epidemiological studies showing increased risk for development of cognitive impairment and AD in patients with various CNS infiltrative and systemic chronic infections. Peripheral viral reactivation with resultant systemic inflammation is a potential mechanism involved in the association between HSV-1 seropositivity and cognitive impairment in younger patients ages 6 to 16 as found by Tarter *et al*. [87]. Systemic HSV-1 induced inflammation may also explain the association between HSV-1 positivity, cognitive impairment, and neurodegeneration on MRI in relatively younger schizophrenic patients as demonstrated by Schretlen *et al*. [34]. Younger subjects have been shown not to have HSV-1 in the brain [107] so presumably, the patients in these studies did not have direct HSV-1 CNS infiltration by the virus. Limited studies to date have failed to detect HSV in postmortem brain tissue from patients with schizophrenia as well [268], including one study using nested PCR [269].

The *APOE-ε4* allele with resultant ApoE4 phenotype impacts the pathophysiology of AD by increasing the pathogen load in the brain, specifically for HSV-1 and *C. pneumoniae*. ApoE4 also interacts with pathogens to enhance the human innate pro-inflammatory response and contributes to the breakdown of the BBB.

HSV-1 may be a primary CNS infiltrative pathogen in the pathophysiology of AD. There is substantial evidence for HSV-1 causation in AD involving stud-

ies in epidemiology, neuropathology, and molecular biology as cited in this review. There is the high prevalence of HSV-1 in both elderly normal brains and AD brains [4, 41] (Table 1) and the presence of both HSV-1 in brain and carriage of the *APOE-ε4* allele significantly increases the risk for sporadic AD [49]. Data for both *C. pneumoniae* and spirochetes (Table 3 and Fig. 3) shows a high prevalence of these pathogens in AD brains only [5, 126], suggesting that secondary *C. pneumoniae* and/or spirochete infection of brain may occur after HSV-1 and other co-factors have already initiated AD pathogenesis. Additional studies need to be done to confirm this hypothesis.

A comprehensive antimicrobial treatment strategy for AD must be developed and tested in clinical trials focusing on pre-clinical or early onset AD. Itzhaki [270] and Strandberg [28] have proposed a randomized controlled antiviral clinical trial using oral valacyclovir. The initial study would test the concept that HSV-1 in *APOE-ε4* carriers is causative in AD [80]. Additional AD clinical trials could then evaluate the AD multi-pathogen hypothesis by testing the effectiveness of valacyclovir combined with the appropriate antibiotics used to treat spirochetes, chronic persistent *C. pneumoniae,* and systemic AD associated pathogens such as *H. pylori* and periodontal infections.

Safe, effective, and less toxic treatments for CMV and other *Herpesviridae* must be developed. Antimicrobial medication in combination with anti-inflammatory treatments may also be beneficial in the treatment of AD. Vaccines against CMV, HSV-1, and other *Herpesviridae* must be developed, as reducing primary infection or reactivation may be useful in AD prevention.

Treatment of HSV-1 and other pathogens present in the AD brain and peripherally may be the most efficacious way to reduce CNS inflammation. The goal of such treatment would be to lower production of pro-inflammatory molecules, reduce accumulation of Aβ, and lower the levels of hyperphosphorylated tau proteins. Antimicrobial therapy could decrease neuronal damage and apoptosis and ultimately aid in the prevention and treatment of AD.

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Section 6
Fungal infection and Alzheimer's disease

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Alzheimer's Disease and Fungal Infection

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Abstract. Alzheimer's disease (AD) is a progressive neurodegenerative condition that leads to dementia mainly among the elderly. Despite numerous efforts from many laboratories, the precise etiology of AD remains elusive. We have analyzed the existence of fungal infection in AD patients. A number of tests have been carried out in blood serum, including the detection of antibodies against several yeast species and fungal proteins, and also the presence of fungal (1,3)-β-glucan. Results from this analysis indicate that disseminated fungal infection can be detected in the majority of AD patients tested. We show that fungal proteins can be detected in cerebrospinal fluid using a slot-blot assay with different anti-fungal antibodies. In addition, proteomic analysis provides strong evidence for the existence of fungal proteins in brain samples Furthermore, amplification of fungal DNA by PCR followed by sequencing distinguishes several fungal species. PCR analysis of these samples reveals a variety of amplified DNA fragments that are dependent on the patient and the tissue tested. DNA sequencing of these fragments demonstrates that several fungal species can be found in brain samples. Collectively, these various assays show that fungal macromolecules can be detected in brain from AD patients and direct visualization of fungal infection in brain tissue provides compelling evidence for the presence for yeast-shaped cells and fungal hyphae. To our knowledge, these findings represent the first evidence that fungal infection is detectable in blood and brain samples from AD patients. The possibility that this may contribute to the etiological cause of AD is proposed.

Keywords: Alzheimer's disease, fungal PCR, endomycosomes, cerebrospinal markers, fungal proteomics, brain immunohistochemistry

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia in elderly people and is characterized by progressive memory impairment, with subsequent behavioral disturbances and profound deterioration of daily life activities [1]. It is estimated that there are at present over 30 million AD patients worldwide and this number will increase to about 65 million by 2030 [2, 3]. A number of risk factors have been recognized by several epidemiological studies, with aging being considered as the most important. Atherosclerosis, hypercholesterolemia, obesity and diabetes also increase the risk for AD [3–7]. Several postmortem pathological features are observed in brains from AD patients, including the presence of extracellular deposits of amyloid-β (Aβ) plaques, intracellular neurofibrillary tangles of hyperphosphorylated tau protein, and neuronal loss [8, 9]. Amyloid precursor protein (APP) is a ubiquitous integral glycoprotein that exists as different isoforms depending on the alternative splicing of its mRNA. Three predominant APP molecules are known to exist: APP751, APP770 and APP695. The latter is the predominant isoform in the brain, expressed mostly by neurons, and is the shortest. The two longer isoforms, APP751 and APP770, are expressed predominantly in glial cells such as astroyctes [10]. Aβ is generated by proteolytic processing of APP through the amyloidogenic pathway, generating a peptide of

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39–42 amino acid residues [8]. APP synthesis, trafficking and metabolism can produce either the toxic Aβ peptide *via* the amyloidogenic pathway, or the sAPPα fragment *via* the non-amyloidogenic pathway [10]. In normal neurons, tau protein serves to stabilize microtubules by a mechanism involving its phosphorylation and dephosphorylation [11–13]. Hyperphosphorylated tau protein polymerizes and is unable to interact with microtubules, leading to the generation of neurofibrillary tangles, which are harmful for cells [14]. These tangles are most abundant in the cortex, hippocampus and amygdala [15]. The number and distribution of cortical tangles correlates with cognitive decline and is the essence of the hypothesis that Aβ increases tau phosphorylation, triggering cell death and AD.

Although most AD cases are sporadic, a subset of cases (around 1-2%) has an early onset of the disease and usually presents mutations in three genes: *APP*, *presenilin* 1 and *presenilin* 2 [16, 17]. In the late onset form of the disease, which is by far the most common, the best-established genetic risk factor is the association with the E4 allele of apolipoprotein E (ApoE4) [18–20]. ApoE is involved in the mobilization and redistribution of cholesterol in the periphery and also during neuronal growth and repair [18, 21]. It is thought that in addition to these four genes, multiple genetic factors govern the susceptibility to AD [16, 17, 22].

Cerebrovascular lesions, including hemorrhages, microinfarcts and vascular degeneration, are observed in 60–90% of AD patients. These vascular disorders can contribute to cognitive decline and the underlying pathology of the disease [23–26]. Indeed, systemic inflammation is observed in AD patients, including elevated levels of proinflammatory cytokines and also the presence of complement components in amyloid plaques [27], leading to the consideration that AD has an autoimmune component [27, 28]. In addition to autoimmunity, other hypotheses have been put forward to explain the different clinical symptoms of AD. Among these, one of the most accepted theories is the "amyloid cascade hypothesis" [2] as indicated above. According to this hypothesis, the initial symptoms of the disease can be explained by the deposition of Aβ that is produced by an imbalance between its production and clearance. This hypothesis, however, fails to explain several clinical symptoms of the disease and has been questioned by several researchers [29].

A number of infectious agents have been suspected to be the etiological cause of AD. Among these, Herpes viruses and bacteria have been suggested as the triggers of the disease [21, 30–33]. To the best of our knowledge, fungal infection has not been considered as the etiological agent of AD, although interestingly some patients with fungal infections have been misdiagnosed with AD [34, 35]. During the course of our investigations on the presence of fungal infection in patients diagnosed with acute zonal occult outer retinopathy and multiple sclerosis, we developed several techniques to detect and measure this type of infection in blood serum [36–40]. We review here the evidence that AD patients exhibit clear signs of fungal infection.

Our knowledge on AD is consistent with the concept of fungal infection as the etiological cause

To our knowledge, none of the clinical symptoms and observations described for AD patients precludes the possibility that the disease may be caused by a mycosis. On the contrary, the observations support the concept that fungal infection could be the etiological agent of this disease. The possibility that infection by fungi exists in AD patients, however, has not been explored by other laboratories. We outline below the important elements that are consistent with this novel concept.

1. AD is chronic and progressive, which concurs with the slow progression and chronicity of many fungal infections if untreated. Besides neurological symptoms, many AD patients exhibit different pathologies, which can also be explained by considering disseminated fungal disease.

2. The pattern of focal lesions observed in AD brains are consistent with an infectious agent.

3. Aβ peptide has antimicrobial activity and is particularly effective against *Candida albicans* [41, 42]. This finding can provide a clue for the physiological function of this peptide and might change our current views about its involvement in AD pathology.

4. Amyloid deposits can be viewed from a different perspective. The actual hypothesis that amyloid deposition may be responsible for the disease is at odds with several observations [29]. For example, strategies aimed to reduce β-amyloid burden have failed to improve symptoms in clinical trials [43, 44]. If fungal infection exists in AD brains, it is possible that Aβ peptide is synthesized as a natural antifungal agent. Accordingly, fungal infection could trigger the synthesis of amyloid as part of the innate immune

response, leading to Aβ deposition that in turn promotes neurofibrillary tangles and neurodegeneration.

5. It is noteworthy that antifungal treatment reverses the clinical symptoms of some patients diagnosed with AD [34, 35]. Indeed, fungal infection was detected after a more thorough analysis of these patients and patient dementia was reversed after antifungal treatment. Nonetheless, the possibility of misdiagnosis of these patients was suggested.

6. Chitin-like material has been detected in brain tissue from AD patients [45–47]. It is plausible that the calcofluor staining method employed to detect chitin identified intracellular fungal cells, although this possibility was not considered in the original studies. It is therefore conceivable that the chitin-like material described in close contact with blood vessels in AD was due to fungal infection.

7. Chitinase (chitotriosidase) levels are increased in the blood serum and cerebrospinal fluid (CSF) of AD patients [48–51]. Presumably, the presence of fungal chitin, the substrate of this human enzyme, induces the production of chitinase.

8. Inflammation and vascular dystrophy are observed in many AD patients [23–26], which is consistent with the established view that fungal infections induce inflammatory reactions as well as vascular modifications.

9. Increased cytokine production, particularly IL-1, has been described in the plasma and CSF of AD patients [52–54]. Indeed, inflammatory proteins in plasma, such as C-reactive protein and IL-6, have been found to be elevated several years before the onset of dementia [55]. Interestingly, fungal infections can elicit the Th1 pathway with production of TNF, IFN-β, IL-1, IL-6 and IL-12, leading to protective immunity. Alternatively, they can induce the production of IL-4 and IL-10, typical of the Th2 response, which is associated with disease exacerbation and pathology [56].

10. Genetic predisposition of a small percentage of AD patients has been established [16]. This is not inconsistent with the possible fungal origin of AD since genetic background may determine susceptibility to fungal colonization [57–59].

11. The presence of APOE 4 alleles constitutes an important risk factor for AD [19], and is also associated with an increased risk for microbial infection [21].

12. The severity and evolution of clinical symptoms in each AD patient varies, which is consistent with the possibility that different fungal species are involved in the etiology of AD. Thus, combinations of different species infecting a single patient may have repercussions for the velocity of cognitive decline and can explain the variety of other clinical symptoms.

Aside from these general considerations, we have directly investigated the potential fungal infections in AD patients. Our results indicate that there are indeed fungal macromolecules in blood serum, CSF and brain tissue in these subjects.

Development of assays to analyse fungal infection

Because a universal test to accurately determine the existence of disseminated fungal infection does not exist, the preferred method to investigate potential infections in patients diagnosed with AD would be to perform several complementary assays, including the detection of antifungal antibodies and fungal macromolecules. Initially, we examined peripheral blood serum for: 1) antibodies against different *Candida* spp., 2) antigens from several fungal species using a slot-blot technique with several rabbit polyclonal antibodies raised against different yeasts, and 3) the presence of fungal polysaccharides, specifically (1,3)-β-glucans measured with the Fungitell® assay (Associates of Cape Cod, Inc.). We next tested the levels of fungal proteins and DNA in CSF from AD patients, and proteins from brain samples were analyzed by SDS-PAGE and mass spectrometry. The presence of fungal DNA in brain tissue was measured by PCR. Finally, we directly visualized fungal structures in brain samples from AD patients. The age and gender of the different patients and control subjects analyzed in this work are listed in Table 1.

Evidence of fungal infection in peripheral blood

Initially, we analyzed the levels of antibodies against different fungal species in blood serum from AD patients [60]. A clear patient-related variability in the detection of anti-*Candida* antibodies was evident in this group. Notably, some of the patients presented a wide and robust immunoreactivity against the majority of *Candida* spp. tested, whereas other patients had almost no antibodies against the yeast species analyzed. Moreover, in some instances a high antibody reactivity against one particular *Candida* spp. could be demonstrated, but not to others. Aside from this patient variability, the important conclusion from this analysis was that antibodies against *Candida* spp. could be detected in blood serum from some AD patients, indicating that they elicited a good

Table 1
Summary of patients used in this study

PATIENT	AGE	GENDER	SAMPLE	
AD1	91	F	CSF	PCR
AD2	80	M	CSF	PCR
AD3	74	F	CSF-BRAIN	PCR,PRO
AD4	93	F	CSF	PCR
AD5	86	M	CSF	PCR
AD6	88	F	CSF	PCR
AD7	69	F	BRAIN	PCR
AD8	82	M	BRAIN	PCR
AD9	74	M	BRAIN	PCR
AD10	82	M	BRAIN	PCR
AD11	69	F	BRAIN	PCR
AD12	70	M	BRAIN	PCR, PRO
AD13	66	F	BRAIN	PCR
AD14	95	F	BRAIN	PCR
AD15	90	F	BRAIN	PRO
AD16	77	F	BRAIN	IMH
AD17	83	F	BRAIN	IMH
AD18	80	F	BRAIN	IMH
AD19	84	F	BRAIN	IMH
AD20	79	F	BRAIN	IMH
AD21	92	M	BRAIN	IMH
AD22	81	M	BRAIN	IMH
AD23	62	M	BRAIN	IMH
C1	64	M	CSF	PCR
C2	43	M	CSF	PCR
C3	58	F	BRAIN	PCR
C4	45	M	BRAIN	PRO

humoral response to several species of the *Candida* genus. An additional feature of this analysis was that not only did the presence or absence of antibodies vary from patient to patient, but also the yeast species recognized by the different sera. This variability may be dependent on the severity of a potential infection or colonization of mucosae. Clearly, the antibodies present in each patient may have different cross-reactivity against different fungal species.

Our next goal was to assess the existence of fungal antigens in blood serum. We developed a highly sensitive method based on the slot-blot technique to analyze yeast antigens in human sera [40, 61]. Using this method, several yeast antigens were detected with rabbit antibodies obtained after injection of different heat-inactivated yeast species [60]. It should be noted that the antigens detected by the slot-blot corresponded to fungal proteins that were detected with the antibody used. The antigen could be of the same species as was used to raise the antibody or, alternatively, there may be cross-reactivity between different species. The majority of AD patients examined exhibited high values with at least one of the antibodies tested. Overall, these findings revealed the existence of a disseminated fungal infection in the majority of AD patients.

The occurrence of fungal polysaccharides in blood serum is apparent in patients with disseminated mycoses [62, 63]. Accordingly, detection of these macromolecules is employed as a panfungal marker of infection [64, 65]. An advantage of this test is that many different fungal species, with the exception of zygomycetes and Cryptococcus spp., can secrete these macromolecules into peripheral blood. We estimated the presence of fungal (1,3)-β-glucan in blood serum using the commercial test Fungitell®. Of note, the vast majority of AD patients were considered positive in the Fungitell® test [60]. Generally, the quantity of (1,3)-β-glucans in serum from AD patients was found to be quite high and in some patients was above 300 pg/ml. These findings add further support to the idea that there are signs of disseminated fungal infection in most AD patients.

Analysis of fungal infection in CSF from AD patients

We evaluated whether fungal proteins and DNA could be detected in CSF from AD patients. As a first test, we used the slot-blot protocol as indicated above to measure fungal antigens. The majority of CSF samples from AD patients gave high densitometric values with at least one of the antibodies, which were above the cut-off values reported previously [39]. Considering all the results generated, a global p value of 0.0016 and an odds ratio of 8 were obtained [60]. It has to be considered that the levels of fungal antigens in CSF fluctuate during the course of the disease. Elevated levels of these antigens in CSF have been previously reported by our laboratory in patients with multiple sclerosis or amyotrophic lateral sclerosis [39, 66], pointing to the concept that mycoses also exists in these two neurodegenerative diseases. These disseminated fungal infections may contribute as a risk factor for these diseases or can play a part in their etiology.

A second sensitive test for mycosis is the analysis of fungal DNA sequences after PCR amplification. We developed several nested PCR-based assays to amplify the internal transcribed spacer 1 (ITS-1) of the fungal genome [66, 67]. Amplified products can be separated on agarose gels and sequencing of the corresponding PCR fragments establishes the species. The specific oligonucleotide primers and conditions employed in these PCR assays have been described in detail. Special care should be taken to avoid contamination during the DNA extraction and PCR steps. We performed three PCR assays after DNA extraction. After a first-round PCR of the ITS-1

region with external primers, three further rounds of PCR were performed with different internal primers (see scheme in Fig. 1). A typical PCR result is shown in Fig. 1. As shown, no DNA products were amplified from controls for the PCR assay and the DNA extraction method. Also, no reaction products were obtained from control patient CSF, suggesting no skin contamination. The products obtained with the three PCR steps were separated on agarose gels and each individual band was extracted and sequenced. The species identified in each sample are described in Table 2. The species detected are potential human pathogens. Collectively, these data demonstrate that DNA from several fungi can be detected in CSF from AD patients and, more importantly, that the species can be identified using this technique.

Proteomic studies in brains from AD patients

A number of proteomic studies have been recently undertaken using CSF and central nervous system (CNS) samples from control patients and those diagnosed with neurodegenerative diseases [68–70], with an aim to analyze the differences in protein composition, specifically human proteins. In doing so, these studies have provided new biomarkers that might help to understand the evolution of disease and to direct adequate therapies [71–73]. In our analysis, we used three frozen brain samples from AD patients and one control sample. The total number of proteins identified in each sample was 4227 for the control brain (C3) and 3080 (AD7), 3372 (AD-8) and 3655 (AD-9) for the three AD patient samples. A number of proteins common to all three patients and not found in the control were described [67]. These differences may reflect proteins that are present in the disease tissue because of neurodegeneration. Indeed, further investigation on these proteins may help facilitate the development of new biomarkers of this disease.

Given that the major aim of our work was to detect fungal macromolecules in brain samples from AD patients, we analyzed the results from the proteomic study described above against the fungal peptide bank. Notably, several fungal peptides were detected, and from these we selected only those peptides that were unequivocally of fungal origin (Table 3). It was striking that one fungal protein, β-tubulin, was detected with 7 peptides. Additionally, several other peptides corresponding to different fungal proteins were identified. Several of these peptides were present in more than one patient, and importantly, none of the AD-specific peptides appeared in the control sample. These observations strongly support the idea of direct fungal colonization in brain tissue from these patients.

Detection of fungal DNA in brain tissue

A very powerful technique to detect fungal DNA in brain samples is the PCR assay [74]. A possible pitfall of this technique is the potential for contamination as described before, leading to false positive results. Therefore, special care was taken during all steps in the process. The reagents used to extract brain DNA together with those employed in PCR reactions were tested to check that no contamination by fungal DNA existed. Using these strict conditions, we found that DNA obtained from control brain samples as well as PCR controls for DNA extraction and PCR reagents were free from fungal contamination. In some of these patients, samples were obtained from different regions of the brain such as frontal cortex (FC), choroidal plexus, putamen-globus palidus or meningeal membrane. The results obtained by real-time PCR suggested that rather low numbers of fungal genomes were found in brain tissue [67]. These findings were consistent with previous work in blood from patients with systemic candidiasis, where only 0.5 yeast cells could be detected per ml of blood [75]. Evidently, the vast majority of DNA in the brain samples is of human origin, and this should be considered when PCR analyses are carried out because the presence of human DNA can interfere with the amplification of fungal DNA. Also, real-time PCR provides no information about the type of fungal species present. For this purpose, it is more appropriate to employ classical PCR. Since the number of fungal DNA copies is very low, nested PCR is the technique of choice as we have previously reported [38, 39]. Amplification of a sub-set of brain DNA samples using this nested PCR approach is shown in Fig. 1. Notably, amplification of several distinct products with DNA extracted from AD brains is apparent in the majority of the samples tested. Furthermore, each sample differs with respect to the number of products amplified and their relative size. As expected, no amplification was observed in the PCR and DNA extraction controls, and importantly brain control samples were also negative. These findings support the contention that there is indeed fungal DNA in CNS from AD patients. Perhaps not surprisingly, different products are amplified depending on the brain region tested, revealing that there is no

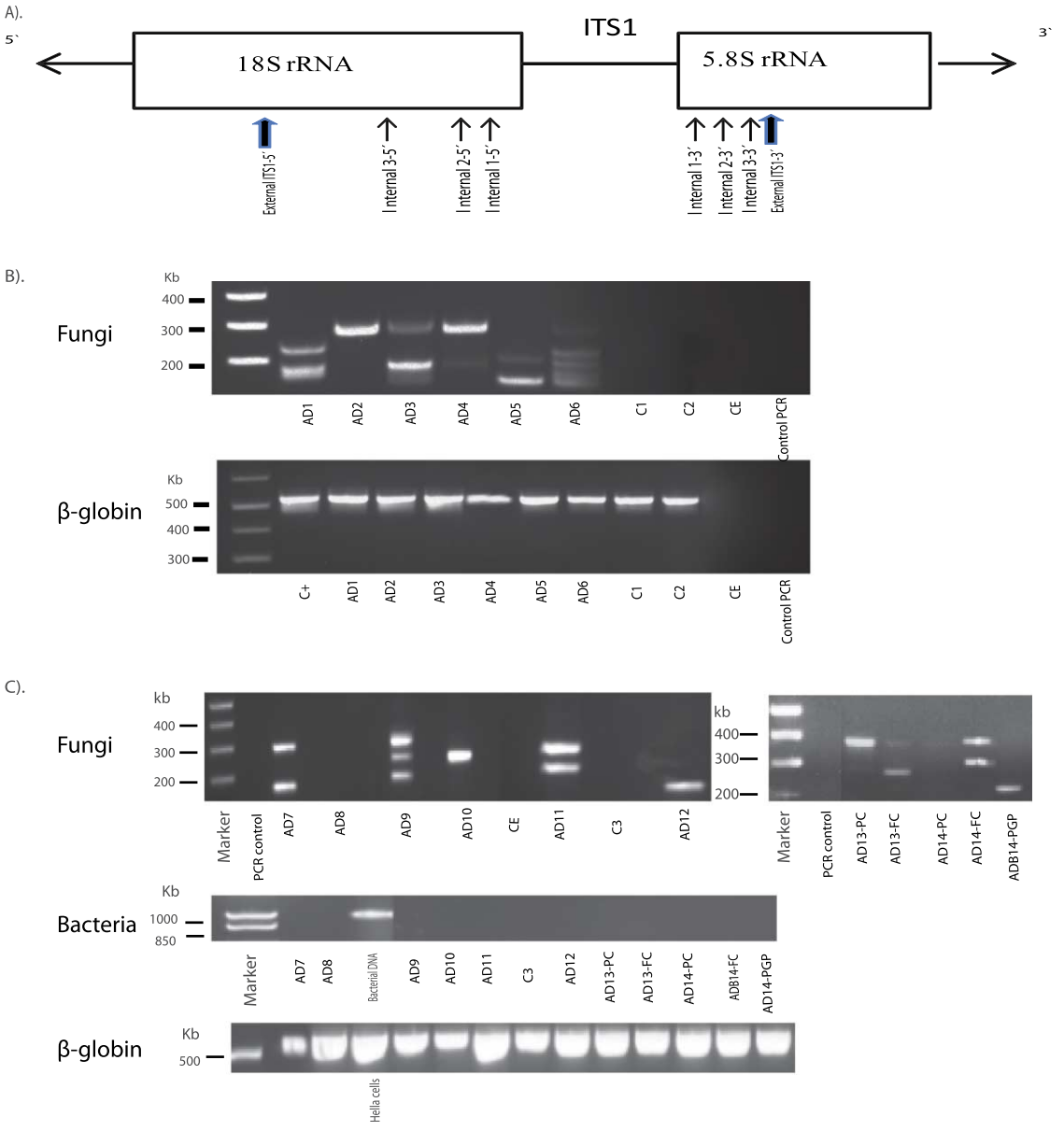

Fig. 1. PCR analysis of DNA obtained from CSF and brain tissue. Panel A: schematic representation of fungal rRNA genes (18S and 5.8S) and the ITS-1 sequence. Location of the primers employed for the different nested PCR: External primers ITS-1 employed in the first PCR, internal primers 1, internal primers 2 and internal primers 3 employed in the different second PCR. Panel B: Analysis of the DNA amplified products by agarose gel electrophoresis. Nested PCR of DNA extracted from CFS of AD patients and controls. The primers employed were primers external ITS-1 for the first round PCR and primers internal 1 for the second PCR assay (fungi; upper panel). PCR analysis of DNA extracted from these samples was also tested using human β-globin oligonucleotide primers (β-globin; lower panel). After PCR, the samples were separated on agarose gels and stained with ethidium bromide. DNA size markers are shown on the left. Control +: DNA HeLa cells. Control PCR: PCR without DNA. CE: Control of DNA extraction without CSF. Panel C: PCR analysis of DNA samples obtained from different frozen brain tissues. Control PCR: PCR without DNA. CE: Control of DNA extraction without brain DNA. a) Nested PCR carried out as in panel B of DNA extracted from brain samples obtained from different AD patients (fungi; upper panel). PCR analysis of different brain regions from two AD patients. PC: choroid plexus. FC: Frontal cortex. PGP: Putamen-globus pallidus. PCR analysis of DNA extracted from the samples tested above using bacteria (bacteria; middle panel) or human β-globin (β-globin; lower panel) oligonucleotide primers. As a positive control, bacterial DNA was extracted from *Thermus thermophilus*.

Table 2
Fungal species present in CSF and brain tissue from AD patients detected by PCR

Species	AD 1	AD 2	AD 3	AD 4	AD 5	AD 6	AD 10	AD 11	AD 12	AD 13	AD 14	AD 15	AD 16	AD 17
Candida albicans	2;3			2	1	1								
Cladosporium				3										
Cryptococcus	1		1;2	1		2								
Malasezzia globosa		1	1	1			1		1	1	1	1	1	1
Malasezzia restricta	1					1			1		1			
Penicillium sp														1
Phoma							1					1		
Saccharomyces cerevisae						3							1	1

Numbers refer to the PCR amplification schedules that were positive for a given species. PCR 1: External ITS1 + ITS-1 (internal 1). PCR 2: External ITS1 + ITS-1 (internal 2). PCR 3: External ITS1 + ITS-1 (internal 3).

Table 3
Fungal peptides present in brain tissue from AD patients

Protein	Accession number (Uniprot)	Peptides	Xcorr (AD 7, AD 8, AD 9)
	Q534E6;Q534E7;Q534F9;Q534F6	AILVDLEPGTMDTIK	4.23, —, 3.78
	Q9P334;F4P7H8;Q86ZX3;Q2TTD3;Q86ZX5; Q7Z861;Q86ZX0;Q86ZX1;Q29TI9;Q29TI2; Q29TI8;Q9P8Z3;Q534F7;Q86ZW9;F4NUL6; Q29TG5;Q9P8Z0;Q29TJ2;Q7Z8C6;Q29TI1; Q9P8Z2;Q7Z8C7;Q29TI3;Q2TTD1;P30668; Q29TI6;Q29TI7;Q29TI4;A0N0G9;D8Q773; Q2TTD2;	MSVTFIGNSTAIQELFK	3.98, —, 4.26
	Q6X0Q0		
β-tubulin	Q534E6;Q29TM2;Q29TN5;Q29TL3;Q9UUP0; Q29TR1;Q29TL2;Q29TM7;Q29TM9;Q29TN4; Q29TP9;Q29UP4;Q29TJ4;Q29TQ1;Q29TN9; Q9UUP1;Q5IW30;Q29TN2;Q29TR2;Q29TP8;	MTSTFVGNSTAIQELFK	—, 4.21, 3.93
	Q29TN7;Q29TQ8;Q29UP6;Q9P8Z8;Q29TM0; Q534E7;Q9P8Z1;Q534F9;Q29TM5;Q29TL8; Q29TP3;Q29TL9;Q29TP2;Q29TQ7;Q29TL6; Q29TP6;Q29TP4;Q29TN1;Q29TN0;Q29TM3; Q29TQ0;Q29TL1;Q29TM1;Q534F6;Q29TN6; Q29TQ5;Q29TM6;Q2QJT0	MSGTFIGDSTAIQELFK	5.07, —, 3.74
Actin	C6GJC9	SYELPDGQVITIGDER	3.46, —, 4.70
	A8PB07	TYELPDGQVITIGNER	—, 3.59, —
Malate dehydrogenase	P17505;Q6CIK3;A6ZZN3;E7LWT3;C5E3W9; C5DDI2	VTVLGAGGGIGQPLSLLLK	—, 4.26, 5.27
HSP70	A1XM63;Q2TTE6	IINEPTAAAIAYGLDQK	3.76, —,3.89

uniform distribution of the different fungal species in the brain tissue [67].

An obvious advantage with classical PCR is the possibility to sequence the amplified products to determine the precise fungal species. By doing so, we detected the fungal species listed in Table 2. It should be noted that some species were prevalent in several samples, while others were detected only in one sample. Therefore, consistent with our results from proteomic analysis, a clear variability occurs regarding the number and particular species present in each sample. These observations point to the idea that there may be a mixed fungal infection in CNS from AD patients. This variability in the fungal species detected may account for the different severity of clinical symptoms and the evolution of the disease in different patients.

Direct visualization of fungal infection in brain AD by immunohistochemistry

Our main goal was to directly visualize the presence of fungal components in brain tissue from AD patients using antifungal antibodies. It must

be considered that these antibodies were obtained using whole yeast cells comprising many proteins and also polysaccharides, and thus cross-reactivity may occur between related fungal antigens. Notably, double immunofluorescence staining of AD brain sections using anti-tubulin and anti-*C. glabrata* antibodies demonstrated the presence of microtubules in the cytoplasm, and also small punctate bodies that immunoreacted with the antifungal antibodies and seemed also to localize in the cytoplasm [76]. This morphology was not observed in control brain sections, demonstrating that anti-*C. glabrata* antibodies do not recognize any component in human brain. Indeed, the morphology observed was reminiscent of the staining produced after infection of mice with *C. glabrata* [76]. These fungal bodies are known as intramycosomes or endomycosomes [76, 77]. The immunopositive material was not present in all neurons. The existence of this intracellular material was only apparent using specific antibodies, perhaps explaining why it has not previously been reported, although some similarities with chitin staining are clear [46].

We have extended this analysis and tested FC tissue from several patients [76, 78]. The particular morphology found with anti-*C. glabrata* antibodies varied from patient to patient. Thus, intracellular bodies of about 1 micron in diameter were evident, but the number and intracellular distribution of these immunoreactive bodies differed.

Our previous results suggested that AD patients may present different fungal infections, and also that mixed fungal infections can occur in the same patient. We therefore assessed whether FC tissue from AD brains immunoreacted with antibodies raised against other fungal species. To this end, we employed rabbit polyclonal antibodies against *Penicillium notatum*, *Syncephalastrum racemosum*, *C. albicans* and *C. parapsilosis*. Clearly, these antibodies can recognize different antigens, but it is also possible that the recognition of some fungal antigens may be common to all fungi. As before, no immunoreactivity was observed in control brain sections [78], indicating that potential antigens for the rabbit polyclonal antibodies were absent in neural cells. Interestingly, the *P. notatum* antibody detected small intracellular bodies apparently located to the cytoplasm surrounding the nucleus in a few neurons (Fig. 2). This punctate morphology was absent in neighbouring neurons, further supporting the notion that only a few cells are infected. Additionally, punctate bodies immunopositive for the *P. notatum* antibody were detected in a blood vessel (Fig. 2), suggesting that fungi might also infect capillaries. Indeed, it is well established that fungi can directly infect blood vessels, provoking vasculitis in the CNS [79, 80]. The neurovascular inflammation present in the vast majority of AD patients [81, 82] could conceivably be explained by direct fungal infection of blood vessels. Staining with antibodies to *S. racemosum* and *C. albicans* also revealed a number of punctate bodies around and inside neurons (Fig. 2). Again, many neurons were immunonegative and thus served as an internal control for the specificity of the antibody. As indicated, it is plausible that the different antibody preparations employed immunoreact with different fungal cells or components. If mixed fungal infection exists in a single patient, perhaps the different antibodies detect distinct infected neurons. Alternatively, if there is cross reactivity between these antibodies they could detect common antigens. Regardless, the use of different anti-fungal antibodies serves to highlight the presence of punctate material that exists in some neurons. Thus, these data reinforce the notion that the observed bodies are of fungal origin.

We used confocal microscopy to analyze whether the fungal-related material observed in brain tissue from AD patients was intracellular. Figure 3 illustrates the intracellular bodies and the cytoplasmic distribution in different regions of one AD patient (AD17). It is important to remember that human cells are infected intracellularly by yeast only when they are alive [83], ruling out the possibility that this infection occurs postmortem or during the handling of brain tissue. In conclusion, the fungal-related material resembles that observed in cultured human cells and in the brain of mice infected with yeast cells [76, 83] and it is clearly intracellular. This may be another reason why the detection of this infection is so elusive.

Corpora amylacea contain fungal proteins

The composition of corpora amylacea (CA) has been analyzed in some detail. They mainly contain polyglucans and only a small percentage (4%) corresponds to proteins [84–86]. The exact origin of CA remains enigmatic, but it is thought that they accumulate in elderly people and their formation occurs over long time periods. CA are much more abundant in patients with neurodegenerative diseases and we recently reported that they contain fungal proteins [87]. Indeed, immunohistochemistry analyses using specific antifungal antibodies (anti-*C. glabrata*) revealed the presence of fungal

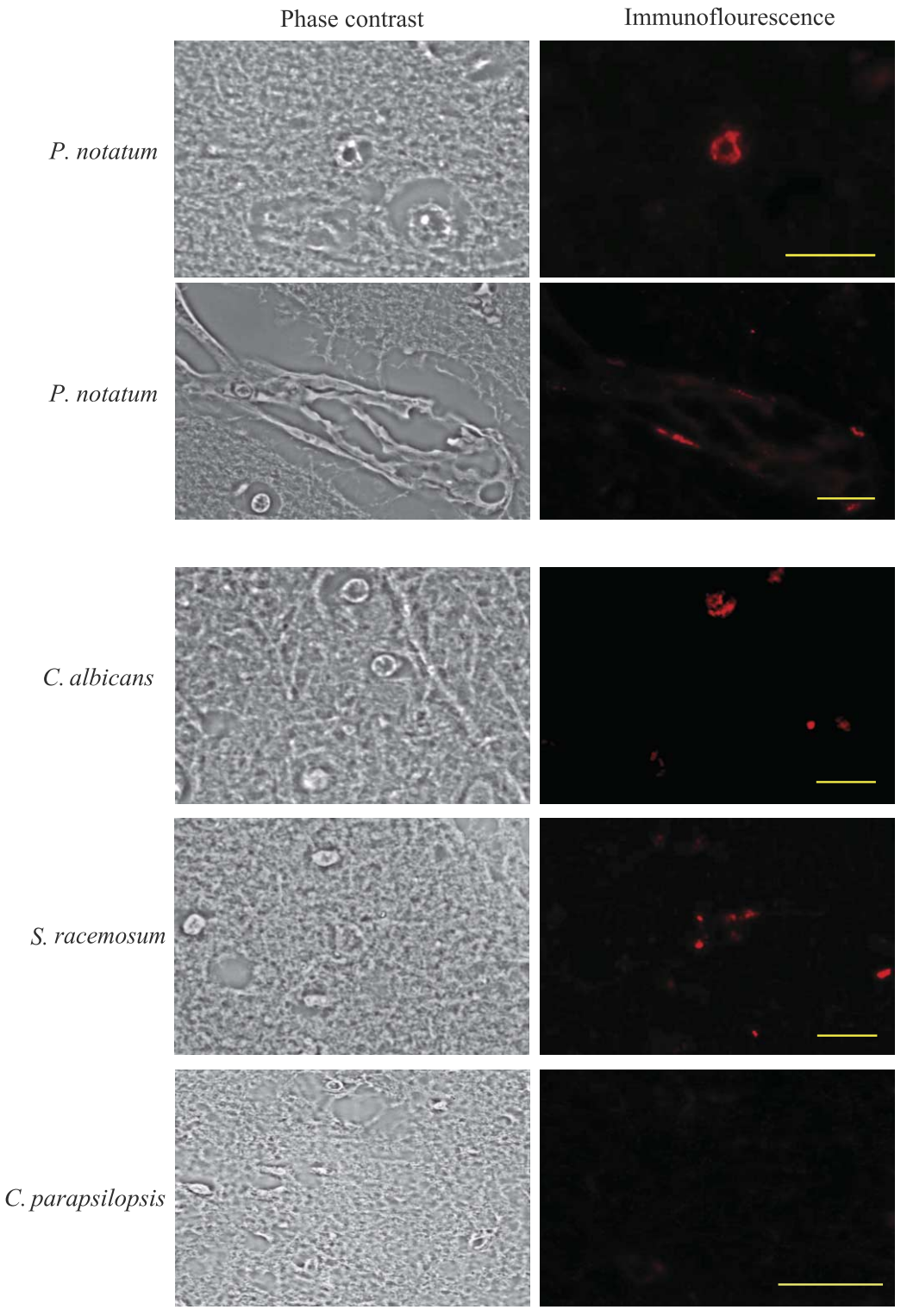

Fig. 2. Immunohistochemical analysis of brain sections from AD patient 16 (AD16) using rabbit polyclonal antibodies raised against different fungi (*P. notatum*, *C. albicans*, *S. racemosum*, and *C. parapsilosis*). Sections were incubated with anti-fungal antibodies (1:500), followed by incubation with secondary antibody donkey anti-rabbit IgG conjugated to Alexa 647 (1:500 dilution). Shown are phase contrast (*left*) anti-fungal antibody (red, *right*). Scale bar: 20 μm. At least 15 different fields were examined (630 × magnification).

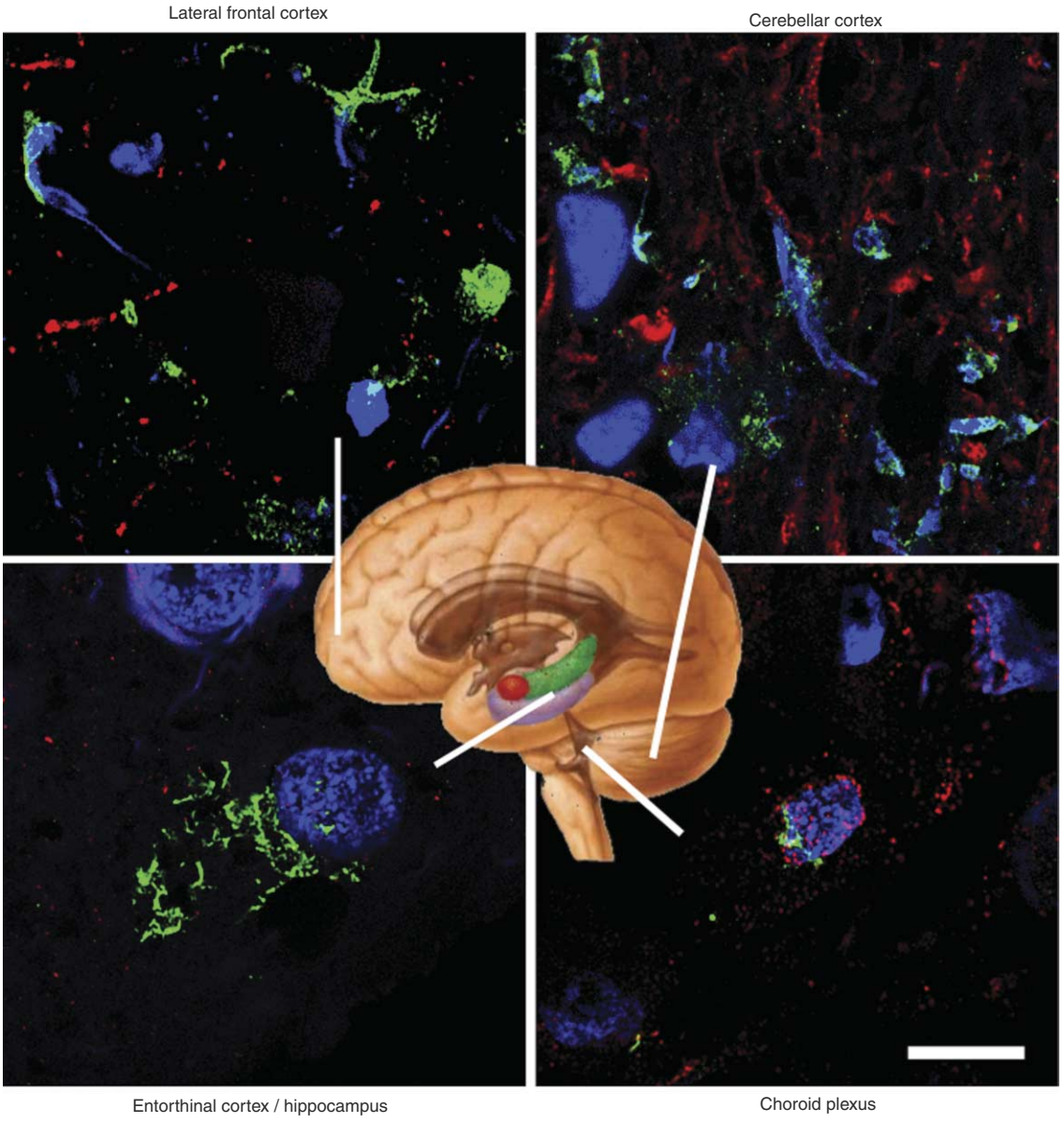

Fig. 3. Immunohistochemistry analysis of tissue sections from different CNS regions using confocal microscopy. Four different regions of the CNS from patient AD17 as depicted in the panels were analyzed by immunofluorescence and confocal microscope. DAPI staining of nuclei is shown in blue. Anti-*C. albicans* antibodies are shown in green. Neurofilaments are shown in red in lateral frontal cortex and cerebellar cortex. Human α-tubulin is shown in red in entorhinal cortex and choroidal plexus. Scale bar: 10 μm.

proteins mainly at the envelope of CA (Fig. 4). This can be observed in different AD patients and in different brain regions [87]. Figure 4 shows fungal proteins in CA from six different AD patients (AD18-23). Double immunofluorescence staining with anti-human α-tubulin reveals that this protein can also be detected, at least in part, in the periphery. Since fungal proteins are incorporated in CA and their formation occurs over long time periods (years), it is very unlikely that these fungal components result from postmortem contamination. Finally, the possibility that fungal infections can play a part in the formation of CA has been recently suggested [87].

CONCLUSIONS

A number of observations from several independent laboratories suggest that a microbial infection

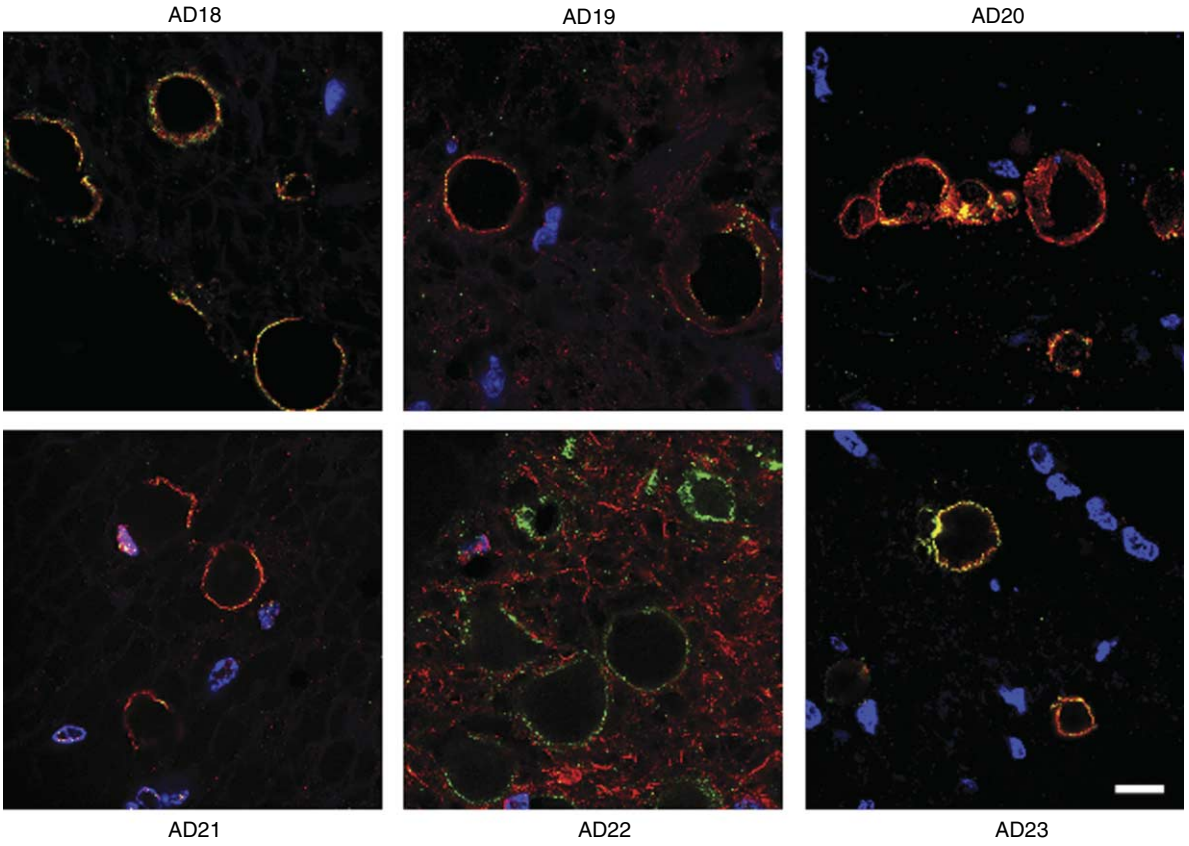

Fig. 4. Detection of fungal proteins in corpora amylacea from AD patients. Brain sections were incubated with anti-*C. glabrata* antibodies (green) and with anti-human α-tubulin (red). DAPI staining is shown in blue. Six different AD patients were tested (AD18-23). Scale bar: 10 μm.

could be responsible for AD. The results obtained by our group provide compelling evidence to support the idea that fungal infection occurs in AD. Thus, analysis of peripheral blood demonstrates the presence of fungal polysaccharides indicating that a disseminated fungal infection is present in AD patients. Moreover, fungal proteins and DNA can be detected both in CSF and brain tissue from AD. Indeed, fungal proteins are detected by proteomic analyses in AD brains and fungal species are evidenced after PCR and sequencing of the amplified products, indicating that mixed fungal infections are taking place. Finally, direct visualization of fungal structures can be accomplished by immunohistochemistry in brain sections from AD patients.

At least two possibilities can be envisaged to explain our results. The first is that for yet unknown reasons, AD patients are particularly prone to fungal infections. The second possibility is that fungal infection is the actual cause of AD. This reasoning, together with the findings reported in the present work lends support to the notion that the etiology of AD may be of fungal origin. To determine whether mycoses are the cause or a consequence of AD, clinical trials with antifungal compounds are needed. Ultimately, these clinical trials could help to determine whether the etiology of AD is of fungal origin, and if this is the case, AD patients may immediately benefit from the use of available antifungal compounds.

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Section 7
Bacterial amyloid, iron, homocysteine, ApoE, and Alzheimer's disease

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Amyloid: Friend and Foe

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Abstract. Amyloidogenesis is the aggregation of soluble proteins into structurally conserved fibers. Amyloid fibers are distinguished by their resistance to proteinase K, tinctorial properties and β-sheet-rich secondary structure. Amyloid formation is a hallmark of many human diseases including Alzheimer's, Huntington's and the prion diseases. Therefore, understanding amyloidogenesis will provide insights into the development of therapeutics that target these debilitating diseases. A new class of 'functional' amyloids promises a unique glimpse at how nature has harnessed the amyloid fiber to accomplish important physiological tasks. Functional amyloids are produced by organisms spanning all domains of life. Understanding how functional amyloid assembly is coordinated will provide new perspectives on what can go wrong when proteins adopt β-rich polymers. Herein we review amyloidogenesis, with special attention focused on the similarities and differences between the best characterized disease-associated amyloidogenic protein, amyloid-β (Aβ), and the formation of several functional amyloids. The implications of studying functional amyloidogenesis and the strategies organisms employ to limit exposure to toxic intermediates will also be discussed.

Keywords: Amyloid, amyloid-β, biofilms, neurodegeneration

INTRODUCTION

Amyloidogenesis is recognized as being the underlying cause of neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's disease. Amyloid fibrils have biochemical and biophysical properties that distinguish them from other biological polymers. Amyloid fibers are incredibly stable, detergent insoluble, β–sheet rich structures that many proteins can form [1]. Amyloid fibers associated with neurodegenerative diseases are considered the product of a protein misfolding event. The pathology of neurodegenerative diseases defined amyloid polymerization as an aberrant process where misfolded proteins aggregate and cause disease. Recent technical advancements have demonstrated the presence of amyloid oligomers both as intermediate and final products of aggregation. These oligomers are believed to be cytotoxic and may play important role in disease progression [2].

However, there are increasing examples of organisms that can utilize either the amyloid fiber itself or intermediates formed during the amyloid polymerization process to fulfill specific biological functions [3–8]. Unlike disease-associated amyloidogenic proteins, functional amyloid assembly is a regulated process that minimizes the cellular toxicity associated with disease-associated amyloids. There are, though, examples where organisms utilize the toxicity of the amyloid fold to kill competing microbes or damage host cells [9]. Understanding mechanisms that promote functional amyloidogenesis will provide an unprecedented glimpse into amyloidogenic systems in general and will lead to new ideas for preventing disease-associated amyloidogenesis. Guided by this perspective we compare and contrast amyloid-β (Aβ)

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amyloidogenesis as it relates to Alzheimer's disease to several systems where functional amyloidogenesis occurs presenting ideas about how these functional amyloid systems prevent the accumulation of amyloid associated toxicity.

AMYLOID AS A LETHAL FOLD

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease. More than 5.4 million people are afflicted with this neurodegenerative disease in the United States alone (www.alz.org./facts). The clinical and neuropathological characteristics were first reported in 1906 by Alois Alzheimer. The abnormal deposits, described as both plaques and tangles, were found in the postmortem diseased brain and were later called amyloid plaques [10]. The plaques were found to be composed of long, unbranched 4–10 nanometer wide fibers when viewed with an electron microscope (Fig. 1A) [11].

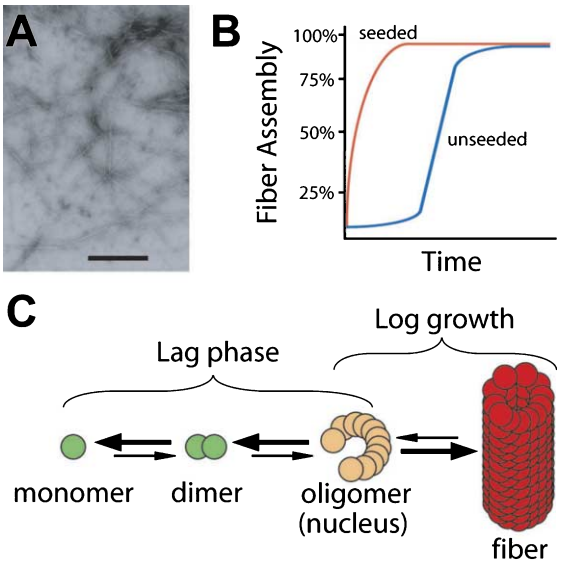

Fig. 1. Properties of amyloid polymerization. (A) Negatively stained electron micrograph of polymerized Aβ fibers. The scale bar represents 500 nanometers. (B) A graphic representation of amyloid fiber polymerization displaying nucleus dependent kinetics (blue line). Preformed amyloid fibers can act as seeds to speed the kinetics of fiber polymerization (red line). This process eliminates the lag phase associated with nucleus formation. (C) Model of amyloid fiber polymerization. A build up of monomer occurs which leads to the formation of multimers and finally the amyloid fiber end product. Large arrows represent processes that are energetically favorable while small arrows represent energetically unfavorable processes.

These structures were discovered to be proteinaceous in nature and contained a uniquely stable cross-beta sheet quaternary structure. Fibers with similar structural characteristics have been described in other neurodegenerative disorders including Parkinson's disease and Huntington's disease [12].

The Aβ polypeptide was purified from AD associated plaques and was determined to be the major protein component of amyloid plaques [13]. Aβ is formed when the amyloid-β precursor protein (AβPP) is sequentially cleaved by β- and γ-secretases [14]. It is proposed that AβPP plays important physiological roles in cell adhesion, neurite outgrowth, synaptogenesis and synapse remodeling [15], however, the exact function of the Aβ polypeptide is still unknown. There are two major cleavage products, $A\beta_{40}$ and $A\beta_{42}$ [16]. The primary sequences of $A\beta_{40}$ and $A\beta_{42}$ only differ in that $A\beta_{42}$ has 2 additional C-terminal residues, Ile^{41} and Ala^{42}. Mutations in presenilins, a central component of γ-secretase, account for most cases of familial AD. These mutations increase the production of $A\beta_{42}$ in both transfected cells and transgenic mice [17]. Another risk factor associated in sporadic AD cases is the apolipoprotein E (APOE) ε4 allele. In cultured neuronal cells APOE4 enhances Aβ production by modulating APP processing [18]. In addition, it was reported that APOE4 also modulates the degradation and clearance of deposited Aβ [19].

Several lines of evidence link AβPP and misfolded Aβ to AD (for review see [20–22]). However, the molecular mechanism behind Aβ misfolding and how this leads to AD remains unclear. Hardy and Selkoe proposed the "amyloid cascade hypothesis" in which the central event in AD development is an imbalance between Aβ production and clearance [23]. This hypothesis remains debatable.

In vitro self-assembly of Aβ polypeptides is characterized by nucleation-dependent polymerization kinetics (Fig. 1B blue line) [24]. During the lag phase trace amounts of dimer, trimer, and eventually, nucleus (oligomer) are formed, which favors rapid fiber formation (Fig. 1C) [25]. As with any dynamic polymerization process where different folding intermediates are present at any one time, Aβ monomer, oligomer, protofibrils (short fibrillar aggregates) and fibrils have been observed using different techniques including atomic force microscopy [26, 27]. Amyloid formation inhibitors such as Congo red and curcumin potentially reduce neurotoxicity by stabilizing the monomeric state of Aβ, thus reducing the amount of oligomer intermediates formed [28, 29].

Therefore, neurotoxicity seems to be linked to aggregation of monomers to higher ordered structures [30–32]. Amyloid laden plaques are often found in post-mortem AD brains, which led to the suggestion that mature insoluble fiber aggregates are the causative agent for AD. However, a wide range of soluble nonfibrillar Aβ forms including dimer, trimer, oligomer, spherical aggregates, protofibrils and mature fibers have been reported to be cytotoxic and effect cortical neurons differently [33–39]. Collectively, this data suggest nonfibrillar intermediates trigger neuropathologies. Therefore, the development of neurodegeneration could be induced by a complicated combinatory effect of several toxic Aβ conformers including the fibers themselves.

Despite evidence that prefibrillar aggregates may be the causative agents AD toxicity, many researchers have reported that mature Aβ fibers can be toxic to cultured neuronal cells [27, 40–42]. Many studies have demonstrated that the amyloid fiber is not a static structure. For instance, amyloid fibers formed from an SH3 domain have dynamic properties, in which molecules can be recycled by a dissociation and re-association mechanism within the fibril population [43]. Therefore amyloid fibrils could provide a reservoir for toxic soluble oligomers, which could trigger the pathology [22]. Under different experimental conditions amyloid fibrils may have different potentials to liberate soluble oligomers, which may be cytotoxic to the cultured neurons. In recent years, several small molecules and structure based inhibitory peptides have been synthesized and tested against Aβ aggregation products [44, 45]. Moreover, the molecular mechanisms behind the initial misfolding events that convert soluble Aβ into an amyloid fiber *in vivo* have not been forthcoming. Perhaps exploring systems where amyloid formation occurs as a natural functional process will provide answers to these questions.

AMYLOID AS A FUNCTIONAL FOLD

The bacterial functional amyloids

Curli

The first example of a functional amyloid fiber was demonstrated in the common laboratory bacterium *Escherichia coli*. *E. coli* and other Gram-negative enteric bacteria produce a functional amyloid fiber called curli (Fig. 2A). These fibers mediate many important physiological functions for the cell. Curli fibers are the major proteinaceous component of the extracellular matrix produced by bacteria during growth in biofilms. Curli also induce a potent host inflammatory response, initiate binding to host cells, and increase the ability of the bacteria to persist within the environment and the host [46–50]. The genetic and biochemical tools afforded by *E. coli* have provided an in depth look at how bacteria control the assembly of amyloid fibers [3].

Biosynthesis of curli fibers is dependent on two divergently transcribed operons, *csgDEFG* and *csgBA*, both of which are under the control of a complex regulatory network [51]. The *csgBA* operon encodes the minor and major curli subunit proteins, CsgB and CsgA, respectively. The stability and secretion of both CsgA and CsgB is dependent on the outer-membrane localized CsgG protein [52–54]. The functions of CsgF and CsgE have not been elucidated, but it is clear that CsgE plays an important role in the stability of both CsgB and CsgA, while CsgF is required for efficient curli biogenesis [3, 55, 56].

CsgA and CsgB are the major and minor subunits of curli fibers. When incorporated into curli fibers, both CsgA and CsgB are detergent insoluble. Cells that do not express CsgB secrete CsgA into the extracellular milieu as a soluble, unpolymerized protein (Fig. 2B). Therefore, *in vivo* both CsgA and CsgB are required for curli biogenesis. However, CsgA and CsgB do not have to be expressed by the same cell for curli assembly to occur. In a process called interbacterial complementation, CsgA secreted from a *csgB* mutant, or donating cell, can be polymerized by CsgB produced on the surface of a *csgA* mutant or accepting cell (Fig. 2B and C) [57]. The ability of CsgB to convert CsgA into an insoluble fiber led it to be designated the curli nucleator protein [58, 59].

CsgA and CsgB are 30% identical and 40% similar at the amino acid level, and each protein has a domain composed of five glutamine-asparagine rich oligopeptide repeats (Fig. 2D). Each glutamine-asparagine rich repeating unit is composed of roughly 20 amino acids and is predicted to form consecutive β–strand loop β–strand motifs that stack perpendicular to the axis of fiber growth (Fig. 2D). Peptides composed of the first, third and fifth oligopeptide repeats of CsgA are amyloidogenic [60, 61]. Mature CsgA protein has been purified, and can self-assemble into curli-like amyloid fibers [3]. *In vitro*, purified CsgA can form amyloid in the absence of CsgB, whereas *in vivo,* CsgA amyloid formation is CsgB-dependent.

CsgA and Aβ *in vitro* polymerization share common features. First, *in vitro* polymerization of both

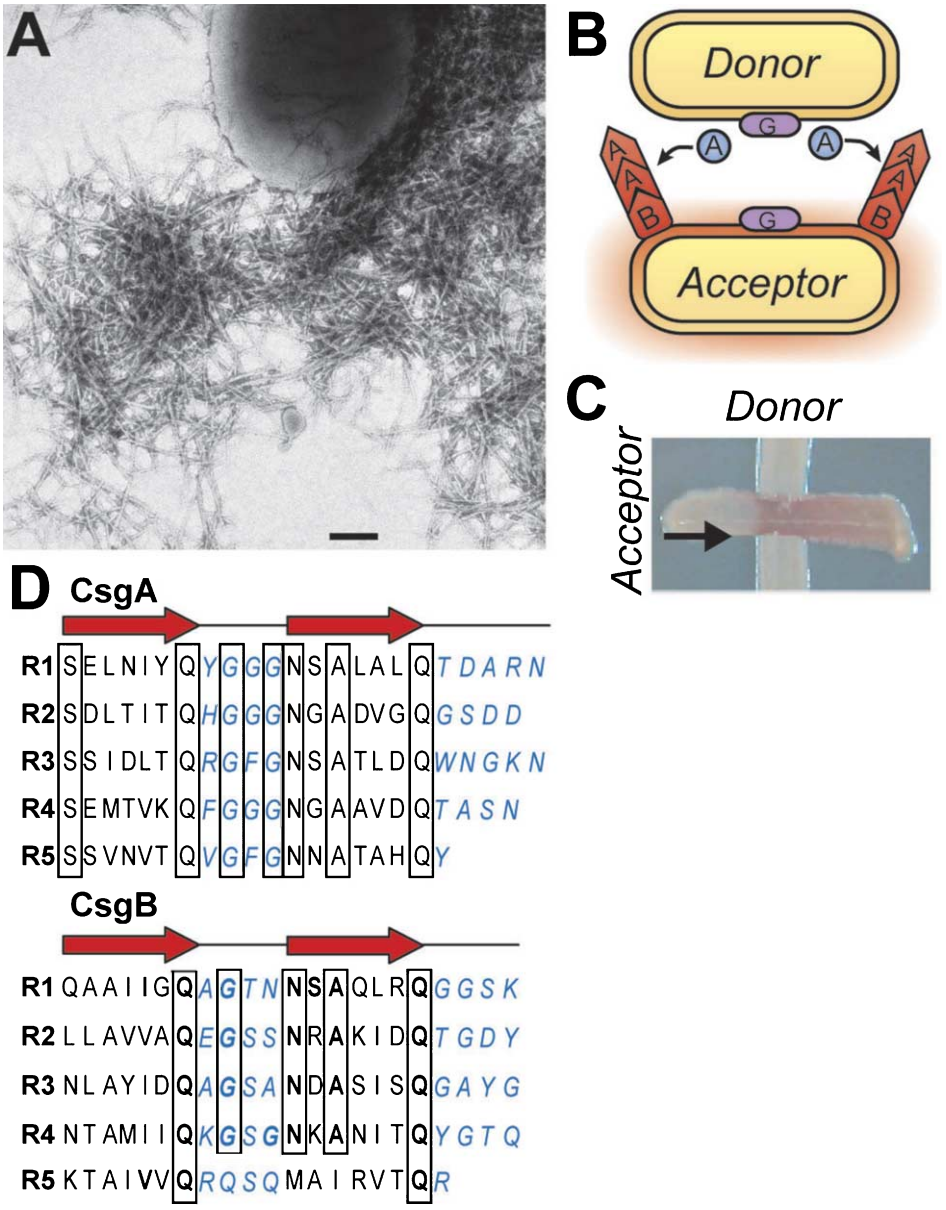

Fig. 2. Interaction between the curli subunit proteins CsgA and CsgB. (A) Negative stain electron micrograph of wild type cells producing curli. Scale bar represents 200 nanometers. (B) Model of Interbacterial Complementation. A donor cells secretes soluble CsgA that acts as a substrate for CsgB on an acceptor cells where curli biogenesis takes place. (C) A Congo red indicator plate demonstrating interbacterial complementation. The donor cells and the acceptor cells appear white until the two strains intersect. Once the two cell types intersect Congo red binding occurs demonstrating curli fiber polymerization as taken place. The arrow represents the direction in which the acceptor cells were streaked onto the plate. (D) The oligopeptide repeating units that compose the CsgA and CsgB proteins. The three dimensional structures of CsgA and CsgB are predicted to be composed of five imperfect β-strand-loop-β-strand oligopeptide repeats (R1-R5). Amino acids comprising the ß-strand are located below the arrows, and amino acids predicted to comprise the loops are denoted with italicized blue letters. Bolded letters represent amino acids conserved in CsgB and CsgA at each position relative to the start of each repeating unit. Boxed letters represent amino acids conserved throughout the repeating units in both proteins.

proteins contains three distinct phases: a lag phase, a growth phase, and a stationary phase (Fig. 1B blue line). Second, in a process called seeding, the lag phase associated with the polymerization of CsgA and Aβ can be abrogated by the addition of pre-formed fibers composed of CsgA and Aβ respectively (Fig. 1B red line) [24, 59]. Lastly, a conformation specific antibody recognizes a folding intermediate

formed during the lag phase of both protein's self-assembly process [62]. These polymerization features are characteristic of an assembly mechanism that is dependent on nucleus formation. Nucleus formation is the rate limiting step and must occur before the protein can begin self-assembly into an amyloid fiber (Fig. 1C). While the roles of nucleus formation as it relates to Aβ polymerization and AD has yet to be elucidated, the curli system is unique in that CsgB's function is to serve as a nucleus for CsgA *in vivo*. A truncated version of CsgB containing the repeating units most similar to those found in CsgA (R1-R4) has been demonstrated to self-assemble into an amyloid fiber (Fig. 2D) [51]. Similar to CsgA and Aβ polymerization, the polymerization of the truncated CsgB resembled nucleus dependent kinetics displaying a lag phase followed by a polymerization phase, followed by a stationary phase. Fibers composed of the truncated version of CsgB abrogate the CsgA's polymerization lag phase, thus recapitulating *in vivo* curli biogenesis [51]. Collectively, these results demonstrate that CsgB acts as a structural template for CsgA polymerization [57, 58].

The separation of nucleation-competent and fiber-forming properties into two proteins suggests that *E. coli* has evolved an elegant strategy to control amyloid fiber biogenesis. A simple model posits that curli biogenesis begins when CsgB is secreted and anchored to the outer membrane where it acts as a template for newly secreted CsgA. In a nucleation-like process, CsgB converts soluble monomeric CsgA into a β–sheet-rich, detergent insoluble protein. Then, in a seeding process, the growing fiber tip can act as a folding template for additional soluble CsgA monomers. By strictly regulating the *csg* operons and by separating the nucleation process and seeding processes into two separate proteins, the cell can ensure that amyloid fiber biogenesis occurs at the right place and at the right time. This strategy decreases exposure to potentially cytotoxic folding intermediates by promoting mature amyloid fiber formation. Therefore, understanding the molecular basis of curli nucleation and polymerization may provide new insights into how the toxicity of disease-associated amyloids can be reduced.

To this end, several studies have been conducted to shed light on the "controlled-chaos" inside the cell that prevents internal amyloid polymerization. Cellular chaperones like DnaK, HSp33 and Spy have been shown to inhibit CsgA polymerization [63]. CsgE, a periplasmic protein that guides CsgA to the outer membrane pore CsgG has also been demonstrated as an efficient inhibitor of CsgA fibril formation [54, 55]. Recent work by Evans et al. has made a significant contribution to the existing knowledge of the curli biogenesis model. The authors discovered that CsgC protein (part of the *csgBAC* operon) is capable of inhibiting *in vitro* amyloid formation by CsgA and helps keeping CsgA in soluble form in the periplasm. CsgC's activity at substiochiometric ratios and without an energy source is striking. This study also suggested that curli production is highly regulated to prevent aggregation inside the cells [64]. These findings suggest that *E. coli* possess orchestrated machinery that exports unfolded CsgA at the right time and place.

Chaplins

The chaplins are extracellular structures produced by the Gram-positive bacterium *Streptomyces coelicolor*. *In vivo*, these amyloid fibers reduce the surface tension at the media/air interface and allow for the development of aerial hyphae [4, 65]. Without the chaplins development of aerial hyphea is impaired. Chaplin biogenesis is dependent upon the translational products of the *chpA-H* operon. Like Aβ and CsgA, the chaplins can assemble into β–rich insoluble fibers that bind the amyloid specific dye thioflavin T *in vitro* [4]. Like curli, chaplin amyloid biogenesis is temporally coordinated. Chaplin expression is dependent on the *bldN* developmental sigma factor, ensuring that fiber formation occurs at the proper time [64]. Furthermore, chaplin amyloid formation is localized to the extracellular space, which may limit exposure to cytotoxic intermediates [4, 64].

Microcin E492 and the harpins

Two examples of bacteria utilizing the cytotoxic properties of amyloid to deter the growth of neighboring cells have been identified. Microcin E492 (also called Mcc), produced by *Klebsiella pneumoniae,* is a potent antibacterial bacteriocin. Mcc is most active during logarithmic growth, losing most of its cytotoxic properties in stationary phase [66, 67]. Bieler and colleagues found that Mcc polymerizes into amyloid fibrils biochemically identical to Aβ and CsgA fibers. Remarkably, the polymerization of Mcc into a mature amyloid fiber coincides with a loss of Mcc antibacterial activity [68]. Thus, a pre-fiber intermediate is proposed to be the cytotoxic species of Mcc [67]. It is also interesting to note that lower concentrations of Mcc are able to induce apoptosis in some human cell lines although the mechanism of Mcc induced apoptosis is currently unknown [69].

The harpins are a second class of bacterial proteins that capitalize on the cytotoxic features of amyloid biogenesis. Produced by plant pathogens, harpins are type-III secreted proteins that induce the hypersensitive response in plants [70]. The hypersensitive response is a plant defense mechanism that slows intracellular pathogen growth by eliciting plant cell death. The hypersensitive response is similar to apoptosis in animal cells [71]. Oh et al. discovered that HpaG, a harpin produced by *Xanthomonas axonopodis* pv. *glycines* 8ra, self assembles into amyloid-like fibers. Unlike Mcc, injection of HpaG protofibrils and mature amyloid fibers into plant cells is toxic and results in cell death. Oh et al. also demonstrated that a harpin from *E. amylovora*, HrpN, and a harpin from *Pseudomonas syringae* pv. *syringae*, HrpZ, form amyloid fibers [69]. Both of these harpins elicit the hypersensitive response. A harpin unable to induce the hypersensitive response, XopA from *Xanthomonas campestris* pv. *vesicatoria*, did not form amyloid fibers [69]. However, a gain-of-function mutant form of XopA (F48L/M52L), which does induce the hypersensitive response polymerizes into an amyloid fiber, correlating the ability to induce the hypersensitive response to the ability to form amyloid [69]. The harpins are an example of a functional amyloid fiber that is designed to be lethal.

MTP

Tuberculosis, one of the most deadly bacterial diseases, is caused by the *Mycobacteruim tuberculosis*. The initial attachment and colonization are the prerequisite for pathogenesis. Prolonged culture of *M. tuberculosis* results in the production of 2-3 nm wide dense, fibrillar aggregates that extend away from the cell surface. These structures were named *M. tuberculosis* pili (MTP) [72]. Alteri *et al.* have experimentally shown that MTP act as an adhesive factor that binds to extracellular matrix and contributes to colonization and infection. Interestingly, the *mtp* genes are conserved in several pathogenic mycobacterial species including *M. bovis* and *M. avium*. The conservation of MTP with the pathogenic mycobacterial species provides further support that the function of MTP is to promote cell invasion and virulence [71].

MTP shares similar features with curli including: i) presence of hydrophobic amino acid sequences including glycine and proline residues, ii) insoluble and aggregation-prone fibers, iii) affinity for the amyloid specific dye Congo Red, and iv) morphologically indistinguishable fiber structures [71].

Biofilm formation by *M. tuberculosis* is regulated by dedicated genetic pathways. The importance of MTP to biofilm demonstrated by the finding that the Δ-*mtp* mutant is severely impaired for biofilm formation. Biofilm formation is restored in the Δ-*mtp* mutant when mtp is expressed from a plasmid [73, 74]. The amyloidogenic properties of MTP are conclusive *in vitro*, however, more evidence is required to establish a role for MTP in cell-attachment and biofilm formation *in vivo*. These studies would greatly enhance the hypothesis that MTP contributes to tuberculosis severity [75].

Phenol Soluble Modulins (PSMs)

PSMs are amphipathic α-helical proteins that have diverse roles in the survival of the Gram positive pathogen *Staphylococcus aureus*. PSMs also increase staphylococcal survival, virulence and pathogenesis. PSMs are usually associated with both hospital-and community-based infections caused by *S. aureus.* [76–78]. PSMs were first discovered in 1999 in the laboratory of Seymour Klebnoff where these proteins partitioned in phenol phase during hot phenol extraction resulting in the name PSMs [79].

PSMs have surfactant-like properties and recently they have been shown to be associated with biofilm formation and dispersal in *S. aureus*. Biofilm formation is a crucial event in staphylococcal colonization of the host. A pioneer study by Schwartz et al. discovered that the PSMs can accumulate in extracellular matrix and form amyloid-like fibers within the biofilm community. These fibrillar structures increase the strength of the biofilm and help bacteria survive stressful conditions. This study also highlights a dual role of PSMs under different environmental cues. Under a stationary state of bacteria PSMs aggregate to form fibrillar structures and provide resistance to mechanical disruption whereas the soluble form promotes biofilm dispersal. Thus, PSMs are capable of regulating *S. aureus* biofilm structure and detachment in diverse microenvironments [80, 81].

FapC

Recently, a functional amyloid was discovered by Otzen's group in *Pseudomonas.* FapC (functional amyloid in *Pseudomonas*) is believed to contribute to biofilm formation by *Pseudomonas spp*. [82–84]. FapC shares similar features with *E. coli* CsgA while maintaining its uniqueness. FapC has an N-terminal sequence and three repeat sequences that Gln, Asn, Gly and Ala rich, but are devoid of aromatic residues [81–83]. The repeat sequences are flanked by large

linkers, but the exact function of the linkers remains to be determined. The great work by Otzen and Nielsen will continue to define how these fibers are formed and how they contribute to community behaviours by the bacteria that produce them.

TasA

TasA was described as a functional amyloid in recent years [85]. TasA was originally discovered as being involved in the sporulation process of the Gram positive bacterium *Bacillus subtilis* [86]. Since the discovery, two independent studies showed that TasA is secreted to the medium during stationary phase in addition to being a constituent of the spore. Overexpression of TasA exhibits antimicrobial activity and resulted in the name translocation-dependent antimicrobial spore (TasA) component. Later, TasA was recognized as an amyloid forming protein and a major component of *B. subtilis* biofilm matrix along with extracellular polysaccharide [EPS] [87, 88]. TasA amyloid fibers form the structural backbone of biofilm and promote various functions such as cell communication and surface adhesion in coordination with EPS [84]. TasA polymerization on the cell membrane is mediated by an accessory protein called as TapA (TasA anchoring and assembly protein) [89]. The mechanism by which TapA mediates TasA polymerization on cell envelope remains unclear but recent experiments suggest that a domain found in the N-terminal region of TapA is either involved in processing TasA or triggering a conformation alteration of TasA that promoted fiber formation [90].

In addition to TapA, a small protein BslA also aids TasA polymerization. BslA self-assembles at the air-water interface and forms a hydrophobic coat around the biofilm. Owing to its physiochemical properties, BslA is structurally defined as a bacterial hydrophobin. Apart from its liquid repellent property, BslA is also proposed to be an important component of biofilm formation and function [91, 92]. Taken together, TasA, EPS, and BslA make a robust extracellular matrix which protects the bacterial community under adverse conditions.

Like CsgA, purified TasA readily self assembles into amyloid aggregates. The morphology of aggregates depends on different environmental cues such as pH and hydrophobicity. Owing to its structural properties and a major constituent of matrix, TasA is proposed to be an attractive model to study amyloid formation and to screen anti-biofilm molecules for therapeutic potential [84].

Listeriolysin

Listeriolysin O (LLO) is a pore forming toxin that is required for *Listeria monocytogenes* to escape from phagolysosomes. LLO promotes release of *L. monocytogenes* from the phagolysome by forming oligomeric transmembrane pores. Once escaped *L. monocytogenes* replicates in the host cytoplasm. Therefore, LLO is considered an important virulence factor for *L. monocytogenes* [93]. The crystal structure of LLO demonstrates that the monomer interface is crucial for oligomerization and pore formation in the membrane [94].

LLO is a pH dependent (cholesterol-dependent cytolysin: CDC) which is active at pH < 6. Under acidic conditions the LLO monomers oligomerize to form pores that facilitate bacterial release from the phagolysosome and entry into the cytoplasm. Initially it was assumed that LLO is degraded after the destruction of the phagolysosome due to the neutral pH of the cytoplasm [95]. However, it has been recognized that at neutral pH LLO forms ordered aggregates akin to amyloid fibrils. The aggregate formation might be an alternate mechanism to sequester LLO and prevent further damage to cholesterol-containing membranes [96]. These aggregates bind to the amyloid specific dyes Thioflavin-T and Congo red. The amyloidogenic properties of LLO was further supported by calculations by TANGO software that predicted several regions in LLO have the potential to form β-sheets. Additionally, the C-terminal immunoglobin superfamily domain in the CDC may promote LLO fiber aggregation and amyloid formation [97]. The conformational switch of LLO under different environmental conditions suggests that the protein has evolved to aid the escape of the *L. monocytogenes* from the phagolysosome and then polymerizes into an amyloid to prevent additional cell lysis [98]. The precise intracellular activity of LLO amyloids is still unclear but it has been speculated that amyloid polymerization in the infected cells might contribute to neurological disorders associated with *L. monocytogenes* infection [95]. The dynamic properties of LLO and the requirement of this protein for *L. monocytogenes* infection, makes LLO a key target for immunomodulation and vaccine development [99].

The eukaryotic functional amyloids

The yeast prion proteins: Eukaryotic functional amyloid domains

The [PSI^+], [URE3], and [PIN^+] phenotypes of the yeast *Saccharomyces cerevisiae* are defined by

non-Mendelian inheritance. These phenotypes are transmitted to daughter cells via a conformationally altered amyloid version of the yeast proteins Sup35p, Ure2p, and Rnq1p, respectively [100–105]. Sup35p, Ure2p and Rnq1p all undergo a conversion to an aggregative state that can incorporate soluble protein into an insoluble amyloid aggregate [106–109]. These proteins all contain a glutamine/asparagine (Q/N) rich domain that is essential for [*PSI*$^+$] and [URE3] prion propagation [110–112]. In both [*PSI*$^+$] and [URE3] this conversion leads to the loss of wild type Sup35p and Ure2p function. The ability to confer a phenotype by converting normally soluble wild type protein into an infectious amyloid aggregate defines these proteins as prions.

[URE3]/Ure2p

Ure2p represses the genetic network needed to utilize poor nitrogen sources [113]. When yeast are provided with good nitrogen sources such as ammonia or glutamine, Ure2p binds to the positive transcriptional regulators Gln3p and Gat1p and prevents their translocation into the nucleus [114–117]. Yeast carrying the [URE3] prion have little Ure2p activity, and no phenotypic advantages have been demonstrated to correlate with the [URE3] prion. In addition, Nakayashiki et al. noted that 70 natural isolates of yeast do not carry [URE3], suggesting that the Ure2p prion state is not advantageous in a natural setting [118]. However, the work by Shewmaker et al. demonstrated that Ure2p missing the Q/N domain had substantially reduced stability and activity [119]. Ure2p missing the Q/N domain had reduced steady state levels compared to the wild type protein suggesting the Q/N domain can act to increase Ure2p stability. These data along with the prevalence of the Q/N domain in the yeast proteome, suggest that the Q/N domain, a domain known to promote amyloidogenesis, may also function as a modular protein-stabilizing domain that also initiates protein-protein interactions.

[PSI$^+$*]/Sup35p*

The Sup35p protein is an essential component of the translation termination machinery. In [*psi*-] yeast, Sup35p recognizes stop codons and terminates protein synthesis [120]. In [*PSI*$^+$] cells wild type Sup35p is sequestered in self-assembled amyloid aggregates. Aggregated Sup35p is unable to participate in translational termination, resulting in translational read-through at wild type stop codons and C-terminally elongated proteins. As with [URE3], the [*PSI*$^+$] phenotype is propagated through the community as dividing cells transmit the [*PSI*$^+$] phenotype to daughter cells [121]. Novel work done by True and Lindquist demonstrated that the [*PSI*$^+$] prion is advantageous under several growth conditions and may provide an alternative mechanism for phenotypic plasticity during environmental insult by altering the yeast proteome [122]. In addition, the Q/N domain itself has been estimated to be conserved for several hundred million years [123, 124]. This suggests a strong selection to retain this amyloidogenic domain despite the possible detrimental effects of decreased translational termination fidelity or amyloid associated toxicity. Because some natural isolates of *S. cerevisiae* do not carry [*PSI*$^+$], it has been proposed that the [*PSI*$^+$] phenotype is not under selective pressure [117]. However, these studies are ongoing and the evolutionary impact of the [*PSI*$^+$] phenotype is difficult to assess.

[PIN$^+$*]/Rnq1p*

The [*PIN*$^+$] phenotype is defined by the ability to induce the [*PSI*$^+$] phenotype. The Rnq1p protein was discovered to induce [*PSI*$^+$] [111]. Interestingly, the only known function of the Rnq1p protein is to induce the [*PSI*$^+$] phenotype *de novo*. In addition to Rnq1p, Ure2p and New1p can also can induce [*PSI*$^+$] when over-expressed [100]. Having a protein dedicated to the induction [*PSI*$^+$] suggests that [*PSI*$^+$] maybe advantageous in growth conditions where *rnq1* is expressed. The study determining the prevalence of prions in natural yeast isolates found that the [*PIN*$^+$] prion is present in some of the yeast found within the environment [117].

Regulation and coordination of yeast amyloid formation

Like Aβ and CsgA, Sup35p and Ure2p self-assemble into amyloid fibers *in vitro* [125, 126]. Also like CsgA and Aβ the self-assembly process of amyloid fiber polymerization contains a distinct lag phase that can be eliminated by the addition of preformed fibers composed of the respective protein [125, 127, 128]. Moreover, like Aβ and CsgA a conformational specific antibody reacts to an intermediate formed during Sup35 polymerization [124, 125]. The curli proteins, CsgB and CsgA, are also similar to the yeast prion proteins, Sup35p, Ure2p, and Rnq1p, in that they all contain the Q/N rich domains [129, 130]. In fact, the GNNQQNY peptide found within the

Q/N rich domain of Sup35p forms biochemically distinct amyloid fibers [131]. This peptide has been used to examine the cross-beta structure of amyloids at high resolution using X-ray crystallography [132]. Also like CsgA and CsgB, oligopeptide repeats are found within the Q/N domain of Sup35p that aid the propagation and amyloidogenecity of the [PSI^+] phenotype [129]. The Q/N domain of Rnq1p also contains several imperfect oligopeptide repeat sequences that are important for the propagation of [PIN^+] [133]. In addition to Sup35p, Ure2p, and Rnq1p, 104 other polypeptides in the *S. cerevisiae* proteome contain Q/N rich domains [134]. However, their ability to form amyloid has not been demonstrated.

The conversion to the prion state for each of [PSI^+], [URE3] and [PIN^+] occurs at a low rate [135]. Even though the conversion rate to the prion state is low, yeast employ molecular chaperones called heat shock proteins to limit exposure to any toxic intermediates formed during prion propagation. Heat shock proteins, such as heat shock protein 104 (Hsp104), play an essential role in modulating the prion state. Propagation of [PSI^+], [URE3], and [PIN^+] all require Hsp104p as deletion of Hsp104 'cures' (i.e. the prion phenotype no longer propagates to daughter cells) cells from [PSI^+], [URE3] and [PIN^+]. However, overexpression of Hsp104p also cures [PSI^+] and overexpression of Ydj1p, a member of the Hsp40 family of proteins, cures cells of the [URE3] prion [136, 137]. Shorter and Lindquist reconciled these seemingly contradictory findings by demonstrating that *in vitro* low concentrations Hsp104 catalyzed the formation of intermediates critical for Sup35p and Ure2p fiber formation, while high concentrations of Hsp104 completely abrogated the ability of both proteins to form an amyloid [111, 124]. These data suggest that Hsp104 may sequester toxic intermediates as well as decrease the time cells are exposed to such intermediates. These findings also suggests that another mechanism heat shock proteins employ to limit exposure to toxic folding intermediates is to speed the formation of an amyloid fiber to the fiber final product.

Filamentous fungi het-S amyloid

Vegetative cell fusions occur within and between individual cells of the filamentous fungi *Podospora anserina*. These fusion events lead to cytoplasmic mixing and the production of a vegetative heterokaryon or multinucleated cells. The *het* locus controls the viability of the fused fungi, whereby heterokaryons that differ at the *het* locus are destroyed. This process is called heterokaryon incompatibility [138]. The *het* locus has two alleles, *het-s* and *het-S*. Het-S is the soluble protein product of the *het-S* loci while the protein product of the *het-s* loci, Het-s, has the ability to convert to an aggregated prion state. When fusion between a *het-S* cell and a *het-s* cell occurs the aggregated Het-s interacts with soluble Het-S and this interaction induces the incompatibility reaction. This leads to death of the heterokaryon and prevents any fusion from occurring between the two cells. Maddelein et al. demonstrated that the heterokaryon incompatibility reaction is induced when cells are transformed with amyloid fibers composed of recombinant Het-s. The incompatibility reaction is not induced when a soluble version of Het-s was transformed into cells. This result provided direct experimental evidence that strengthened the protein only prion hypothesis [139]. The molecular mechanisms of Het-s-induced cell death are currently unknown.

CPEB

CPEBs are highly conserved RNA-binding proteins localized at neuronal synapses that stabilize messenger RNA molecules [140]. CPEBs have been found to be important for memory retention due to their ability to activate dormant messenger RNA transcripts near neuronal synapses. These activated messages can then be translated into proteins that stabilize neuronal synapses or help create synaptic connections necessary for long term memory [141]. The CPEB protein of the sea hare, *Aplaysia californica* (ApCPEB), contains a Q/N rich domain. Si *et al.* demonstrated that ApCPEB acts as a prion in yeast and that the aggregative amyloid state of CPEB is the functional, RNA-binding, form of the protein [142]. Thus, ApCPEB is functionally active when polymerized into amyloid aggregates, whereas, the wild type functions of Ure2p and Sup35p are impaired when the proteins are aggregated. Drosophila has a homolog of CPEB called as Orb2 which also shares structural traits with pathological amyloids [143]. Recent experiments provide evidence of existence of neuronal CPEB in different organisms and the importance of its prion-like state in memory stabilization [144].

Pmel17

Melanocytes and retinal pigment epithelium are specialized cell types responsible for the production

of melanin, a tyrosine based polymer that protects the mammalian eyes and epidermis from ultra-violet damage and other environmental insults. These cells types synthesize melanin within specialized membrane enclosed vesicles called melanosomes [145, 146]. Melanosome maturation and polymerization of melanin are dependent on insoluble fibers composed of the PMEL17 protein [147, 148]. Fowler and colleagues demonstrated that fibers composed of PMEL17 contain the biochemical hallmarks of amyloid [7]. PMEL17 amyloid fibers function by kinetically enhancing the polymerization of melanin, presumably by acting as a scaffold for reactive melanin precursors [7]. Mammalian cells have evolved several mechanisms to reduce exposure to toxic folding intermediates produced by PMEL17 amyloid polymerization: (1) polymerizing the amyloid fiber in a membrane enclosed vesicle sequestering folding intermediates from the cytoplasm, (2) regulating the start of polymerization via proteolysis, and (3) using reaction kinetics that skew towards the stability of the mature amyloid fiber [7, 8].

Peptide hormones

Pioneering work from Roland Riek's lab demonstrated that peptide/protein hormones adopt an amyloid-like conformation [149, 150]. Exocrine and endocrine cells store proteins and peptides in secretory granules for long duration that induces the formation of stable amyloid fibers [151]. These hormone fibrils display all the features of typical amyloids. Interestingly, the fibrils disaggregate into monomers that carry out the function of the specific hormone. It was proposed that the critical concentration and/or processing of (pro)hormone leads to aggregation in Golgi complex. This initial amyloid trigger appears to be sequence specific but once started it acts as seed and directs coaggregation of different hormones. During this process, the aggregated hormones are surrounded by membrane to form a mature secretory granule, partitioning the hormone amyloids from the rest of the Golgi complex. The compartmentalization of the amyloid-like aggregated hormones serves as a storage unit for long durations. These fibrils, though toxic to cells when added to cells grown *in vitro*, circumvent cytoxicity via the encapsulation process. Upon receiving suitable signals, the aggregates disassemble into monomeric entities and are released from the granules [149]. Defining the molecular mechanisms that promote the disassembly of the hormone fibrils could lend tremendous insight into how cells regulate amyloidogenesis and avoid toxic effects.

CONCLUDING REMARKS

Despite over twenty years of AD research the nature of the toxic species of Aβ has yet to be conclusively identified and little is known about how Aβ polypeptide aggregation begins *in vivo*. Insights into these two critical phenomena will undoubtedly lead to advances in AD treatments. The functional amyloids may hold the key to understanding the molecular mechanisms of amyloid fiber toxicity and initiation of amyloid fiber polymerization because they are naturally occurring directed polymerization processes. Not only are the amyloid fiber end products in both AD and the functional amyloid systems biochemically similar, but a common intermediate has been identified for CsgA, Sup35p, and Aβ polymerization. This suggests that amyloid biogenesis occurs via a conversed mechanism. Interestingly, most of the directed amyloid synthesis pathways discussed herein polymerize to higher order aggregates/fibers *in vivo* and these fibers have physiological nontoxic roles in most cases. A folding intermediate of Mcc amyloid fibers is cytotoxic but not the mature amyloid fiber itself. Thus, it seems likely that the functional amyloids lend credence to the hypothesis that the fiber end product may not be toxic. The role of the chaperone Hsp104 in yeast prion propagation also supports this idea. At high concentrations the chaperone abrogates amyloid fiber polymerization, but at low concentrations Hsp104 kinetically accelerates fiber formation. These results demonstrate two mechanisms cells use to sequester the buildup of toxic intermediates. Chaperones either bind the aberrant toxic intermediate, which allows the protein the opportunity to refold, or the chaperone binds to a fiber intermediate and facilitates the conversion to the amyloid form.

These functional amyloid synthesis pathways will continue to provide novel insights regarding amyloid biogenesis. The functional amyloid systems may even address pivotal questions that remain for Alzheimer's disease progression such as, how does amyloid biogenesis begin *in vivo*, what is the most cytotoxic species produced during amyloid biogenesis, and what are the defining features of proteins that preclude the ability to fold into the amyloid conformation. The answers to these questions will in turn provide novel therapeutic strategies for treating disease associated amyloidosis such as AD.

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A Bacterial Component to Alzheimer's-Type Dementia Seen via a Systems Biology Approach that Links Iron Dysregulation and Inflammagen Shedding to Disease

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Abstract. The progression of Alzheimer's disease (AD) is accompanied by a great many observable changes, both molecular and physiological. These include oxidative stress, neuroinflammation, and (more proximal to cognitive decline) the death of neuronal and other cells. A systems biology approach seeks to organize these observed variables into pathways that discriminate those that are highly involved (i.e., causative) from those that are more usefully recognized as bystander effects. We review the evidence that iron dysregulation is one of the central causative pathway elements here, as this can cause each of the above effects. In addition, we review the evidence that dormant, non-growing bacteria are a crucial feature of AD, that their growth *in vivo* is normally limited by a lack of free iron, and that it is this iron dysregulation that is an important factor in their resuscitation. Indeed, bacterial cells can be observed by ultrastructural microscopy in the blood of AD patients. A consequence of this is that the growing cells can shed highly inflammatory components such as lipopolysaccharides (LPS). These too are known to be able to induce (apoptotic and pyroptotic) neuronal cell death. There is also evidence that these systems interact with elements of vitamin D metabolism. This integrative systems approach has strong predictive power, indicating (as has indeed been shown) that both natural and pharmaceutical iron chelators might have useful protective roles in arresting cognitive decline, and that a further assessment of the role of microbes in AD development is more than highly warranted.

Keywords: Alzheimer's disease, bacteria, dormancy, dysbiosis, eryptosis, iron, LPS, systems biology, ultramicroscopy

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INTRODUCTION

Alzheimer's-type dementia (AD) is a neurodegenerative disorder and the most common form of dementia, already in 2013 affecting 44.4 million people globally; this number is expected to affect 75.6 million by 2030 [1]. The current cost is reckoned at $604 billion per year and this figure is expected to triple by 2050 [2]. Due to the increasing prevalence of the condition, the cost to the public health and elderly care systems to support these individuals is increasing exponentially, and posing major financial challenges [3].

Arguably, the major hurdle in understanding AD is the lack of any integrative and comprehensive knowledge about its etiology and pathogenesis (and there may be many pathways that lead to it), as the onset and risk of AD development is still mostly unexplained (and animal models are of questionable relevance) [4]. Since our genomes changed but little in the last 50 years, but the incidence of AD increased considerably [5], this increase can only to a limited extent be explained by genetic factors [6, 7], notwithstanding the signals detectable in twin and gene association studies [8, 9]. Although dementia is properly diagnosed via cognitive impairment, and true diagnoses of AD can only be done postmortem, specific lesions that characterize AD include extracellular senile plaques and intracellular neurofibrillary tangles with synaptic and neuronal loss [10–13]. In particular, the production of senile plagues, a central event in AD [14], is a result of the cleavage of the amyloid-β protein precursor (AβPP). AβPP has important developmental functions in cell differentiation and possibly in the establishment of synapses [15, 16]; however, it is also expressed by neurons in response to cell injury [17]. Neurofibrillary tangles are composed of the tau protein [18]. In healthy neurons, τau is an integral component of microtubules, which are the internal support structures that help transport nutrients, vesicles, mitochondria, and chromosomes from the cell body to the ends of the axon and backwards [19]. In AD, however, τau becomes hyperphosphorylated [18, 20]. This phosphorylation allows tau proteins to bind together and form tangled threads [21], a process that can be reversed by iron chelation [22].

Recent evidence suggests that neuroinflammation may play a major role in the pathological processes of AD progression [23–31]. Indeed, inflammation and microglial activation are known as common components of the pathogenesis of a number of neurodegenerative diseases, including AD, Parkinson's disease, Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis [32]. Several neuroinflammatory mediators, including complement activators, chemokines, cytokines, and oxygen radical species, are expressed and released by microglia, astrocytes, and neurons in the AD brain. While minor signs of neuroinflammation can be found in the normal aging brain, the AD brain faces a much stronger activation of inflammatory systems, indicating that an increasing amount of (or qualitatively different) immunostimulants are present. In recent papers, we have also reviewed the comprehensive evidence that in AD the neuroinflammation is probably a systemic inflammatory condition [33, 34]. In one sense, however, the above are all manifestations or accompaniments of AD, and what we seek are the most important causative pathways. It turns out that central to all of these diseases is iron dysregulation [35, 36].

Figure 1 provides an overview of the article in the form of a 'mind map', while Table 1 lists some of the symptoms (some causative) accompanying the pathology of AD. This wide strategy necessarily involves a systems biology approach [37–41] as we recognize (e.g., [36, 42–47]) that this is the only reasonable strategy for approaching complex biochemical networks, each of whose components may contribute partially to the phenotype of interest.

A typical systems biology strategy (e.g., [42, 43]) has the following four elements: first we identify the actors that are most involved, and how they interact. 'Actors' for these purposes may be enzymes or other biochemical elements, or higher-order physiological processes (such as those in Table 1). We then adduce the order or pathway of such interactions (as in Fig. 2, below). Latterly (though we are not yet ready for this in the present problem), we seek to make quantitative these interactions, and predict their relative fluxes, contributions, and so on. We next turn to some of the main actors, starting with iron dysregulation.

IRON AND AD

Strongly and causatively related to this neuroinflammation in AD is the involvement of unliganded iron and its accompanying oxidative damage in AD etiology [48–61]. Specifically, AD is characterized by elevated brain iron levels [62–64] and the accumulation of copper and zinc in cerebral amyloid-β (Aβ) deposits (e.g., in senile plaques) [65–73].

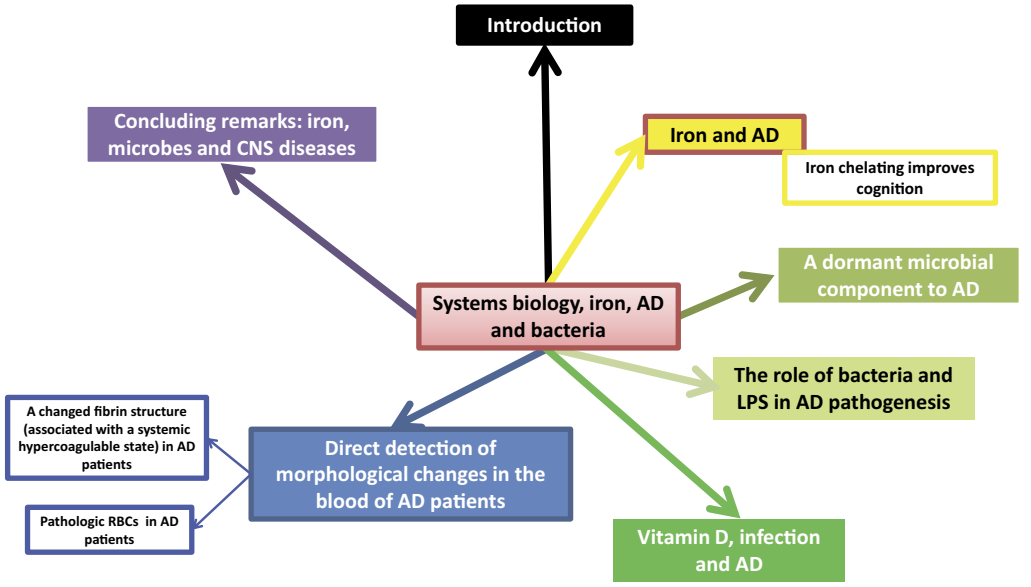

Fig. 1. A mind map summarizing the content of this paper.

Table 1
Some of the most well-known Alzheimer's-type dementia symptoms. Some may be causative

Most well-known (some causative) Alzheimer's-type dementia symptoms
• Pathological loss of microglia, astrocytes and neurons
• Neurofibrillary tangles composed of hyperphosphorylated tau
• Cerebral amyloid-β (Aβ) or senile plaques
• Upregulation of complement activators, chemokines, cytokines
• Reactive oxygen species generation
• Iron dysregulation
• Accumulation of metals in cerebral Aβ deposits (e.g., in senile plaques)
• Neuroinflammation

There is evidence in the literature that the iron status of AD patients, particularly the serum ferritin (SF) levels, as measured systemically, might have clinical relevance, as this is an indication of iron dysregulation [33, 58, 72, 74, 75]. Increased iron levels are also closely linked to hematological pathology in AD, and this is indicative of systemic inflammation, which also plays an important role in the pathogenesis of the condition [54, 76, 77]. Recently, we showed that, in a randomly chosen AD population, 60% of the patients had increased SF levels, causing adverse effect on red blood cell (RBC) structure [33] as well as causing significantly thinner fibrin fiber diameters, resulting in abnormal clotting [78].

Pathology, in the presence of increased SF levels to both RBCs and fibrin formation, is indicative of a systemic inflammatory involvement of iron in AD. In the recent Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort study, increased SF levels were also measured in cerebrospinal fluid and found to be negatively associated with cognitive performance [79]. Systemically elevated SF levels therefore may have great clinical relevance in AD, as they may be useful as markers of cognitive performance.

Currently, the main therapeutic approaches in AD either attempt to prevent Aβ production (e.g., by the use of secretase inhibitors) or to clear Aβ. However, there is convincing evidence that Aβ does not spontaneously aggregate on its own, but that there is an age-dependent reaction with excess brain metal (copper, iron, and zinc), which induces the protein to precipitate into metal-enriched plaques [65]. In AD there is also a dramatic increase in brain iron content and in fact there are higher iron concentrations inside the Aβ plaques [80], suggesting that disturbances in brain iron homeostasis may contribute to AD pathogenesis [81, 82].

It is well known that excessive poorly liganded iron may cause oxidative damage [35, 83, 84], and there is ample evidence that suggests that oxidative stress and

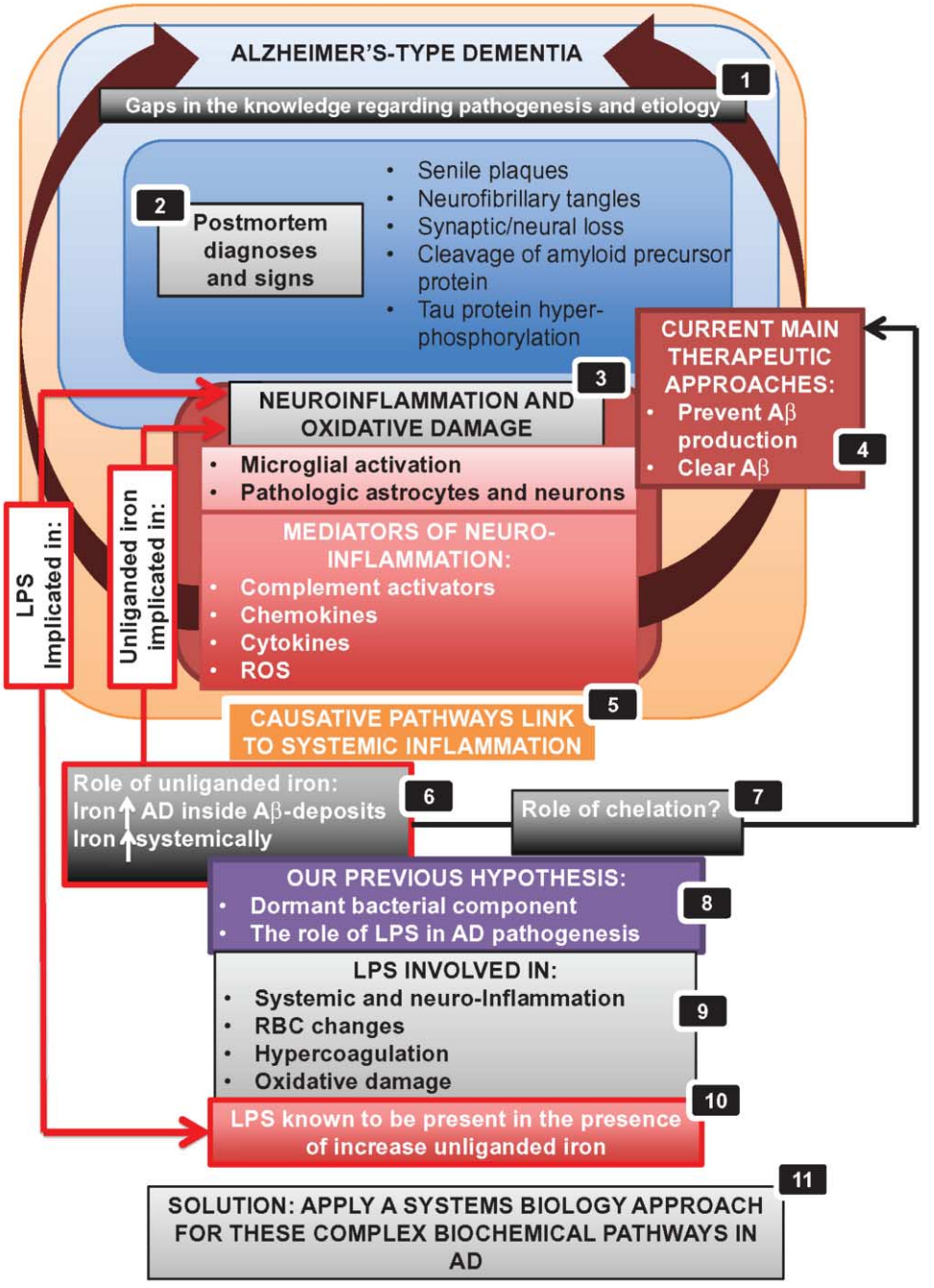

Fig. 2. The order or pathway of major and potentially causative interactions in Alzheimer's- type dementia between enzymes or biochemical elements, following a systems biology strategy.

therefore aberrant redox activity is one of the earliest pathological changes in AD, and that there is a link between systemic and brain oxidative stress [50, 85].

Oxidative stress plays a significant role in the pathogenesis of AD [86–89]. Oxidative stress in AD results in increased levels of lipid peroxidation, DNA, and protein oxidation products (HNE, 8-HO-guanidine, and protein carbonyls, respectively) inside AD brains [90]. Oxidative stress participates in the development of AD by promoting Aβ deposition [91], tau protein hyperphosphorylation, and the subsequent loss of synapses and neurons. In AD, much as

with the prion protein in prion diseases [35, 36, 92], Aβ can become a pro-oxidant and when complexed to iron, this can result in hydrogen peroxide formation; this process can underlie the increased oxidative stress burden [93]. The relationship between oxidative stress and AD suggests that it is an essential part of the pathological process; poorly liganded iron can participate in the Fenton reaction ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH^{\bullet} + OH^{-}$), and the highly reactive hydroxyl radical $OH^{\bullet}$ may be the main culprit [35]. In addition, the Haber-Weiss reaction $Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2$ reverts the Fe^{3+} to Fe^{2+} such that the 'iron' then becomes catalytic rather than stoichiometric [35, 94]; this is why the unliganded iron is so particularly toxic.

In a series of articles, including a number of reviews, we have shown that poorly liganded iron is key to a great variety of diseases [33, 95–97]; it also affects erythrocyte morphology and coagulation properties (touched on briefly later in this paper) [96, 98].

Ultimately, oxidative stress may be due to the combined action of mitochondrial dysfunction, increased metal levels, inflammation, and the presence of Aβ peptides [99]. However, there is a link between all the above-mentioned and the pathological presence of iron. Increased oxidative stress results in inflammation, which can be both neuroinflammation or systemic inflammation [100], and the pathologic levels of iron have been associated with both inflammation and oxidative stress in AD [23, 91]. We tend to like ideas with predictive power (such as unitary explanations for diseases with comorbidities, for which see also [101]). Thus, if iron is so important to the pathogenesis of AD, one might then suppose that its chelation (that stops the Fenton and Haber-Weiss reactions) would be expected to improve it [102, 103]. The next section looks at this.

Iron chelating improves cognition

Starting with a Lancet paper that is now a quarter of a century old [104], it has been shown that the removal of pathologic levels of free iron improves cognitive function in AD. Metal chelators such as clioquinol and desferrioxamine, and natural antioxidants such as curcumin and ginkgo extract, have had some success in altering the progression of AD symptoms [90, 105–107]. More recent and important papers, to the same effect, come from the group of Perry and colleagues [51, 54] and that of Youdim [108], while similar beneficial effects of iron chelation can be observed with Parkinson's disease and models thereof [109–112]. We do find it slightly surprising that these indications have not been more widely picked up.

A fine control of iron regulation might play an important role in systemic iron overload [113] including AD [114], as there is a known association between diet and risk of dementia [115]. Except for pharmaceutical intervention, it is well known that a healthy diet rich in polyunsaturated fatty acids and polyphenols may have a positive effect on general health brain function [116]. In particular, the Mediterranean-type diet has a positive effect on the healthiness of AD patients [117–120], due to the presence of naturally occurring iron chelating agents found in fruit and vegetables as these agents are known scavengers as a result of their ability to chelate iron [118, 121–124]. Another route might also be calibrated phlebotomy in AD, to reduce iron stores [125].

A DORMANT MICROBIAL COMPONENT TO AD

While metals can certainly contribute significantly to the explanation of the development of AD via these Fenton-type pathways, we have recently suggested that they may do so by another and parallel means, explicitly involving the awakening of a dormant bacterial component [34, 126]. This follows from the recognition that the growth of microbes *in vivo* is normally limited by the availability of free iron [127–132]. Others too have noted the presence of an authentic blood microbiome even in 'normal' controls, based on macromolecular sequencing and other molecular approaches (e.g., [126, 133–138]), although sequencing methods cannot of themselves reflect replicative potential, of course.

In this sense, a 'classical', related, and well-known example is that of *Helicobacter pylori* and gastric ulcers. These latter had long been assumed to be due to the over-activity of the gastric H^{+}-ATPase (which can certainly contribute). However, the pioneering (and initially 'controversial') work of Barry Marshall and Robin Warren showed unequivocally that they were inevitably accompanied, and the disease was essentially caused, by a hard-to-culture and little-known microaerophilic organism, subsequently codified as *H. pylori* [139–142]. Our major thesis here (and elsewhere) is that it will turn out that a very large number of chronic, inflammatory diseases, that share many observable symptoms, will also turn out

to be due to hard-to-culture organisms, many or most of which will turn out to be well known to science. The issue is that they typically lie dormant, and thus (by definition) resist culture by means that normally admit their culture.

The point of 'dormancy' is particularly important, as most clinical microbiologists typically consider or define microbial propagules (cells potentially capable of replication) as being 'alive' (i.e., so capable) or not under any conditions tested ('dead'). However, a considerable literature (reviewed by ourselves, e.g., [126, 143–146]) and others (e.g., [147–152]) indicates that most microbes in nature are non-growing and can appear operationally 'dead', yet can recover culturability, by a process referred to (virtually by definition) as 'resuscitation'. They are thus not operationally 'dead' and are typically and more properly referred to as 'dormant' (or, commonly in clinical microbiology, 'persistent' [150, 152–156]). One needs then to recognize that dormancy is an **operational** property that depends both on the cell (singular [157]) being assessed and on the means used to detect it [158]. **This cannot be emphasized too strongly:** the designation of a microbe as dormant implies that it is not just a property of the microbe alone but of the means by which we assess it, a phenomenon reminiscent of the "Schrödinger cat paradox" in the philosophy of quantum mechanics. One important consequence (see e.g., [126, 159–164]) of this ability of microbes to enter non-replicating physiological states is that they do not fulfill the Henle-Koch postulates regarding the microbial causality of diseases, at least in their ordinary form [165].

Particularly, the neurotoxic lipopolysaccharides (LPS) from their cell walls may be of importance (see below), since LPS molecules are highly inflammatory agents, that can even induce cell death [126]. **It is of course the cell death that is the proximate cause of the loss of cognitive function**. We summarize all of these pathways in Fig. 3. The especial attractions of this scheme are that (i) it provides for the necessary systems-level understanding, (ii) the elements hang together and are 'coherent' within the meaning of that term as used in the Philosophy of Science [126, 166], and (iii) it is rich in both predictive and explanatory power.

While recognizing the importance of various kinds of infectious agents in the pathogenesis of AD (see [34, 162, 167–198]), and that also depend for their growth on the availability of free iron, we next turn to the question of the role of prokaryotes and their inflammatory components in the pathogenesis of AD.

THE ROLE OF BACTERIA AND LPS IN AD PATHOGENESIS

Recently, immunoblotting demonstrated bands corresponding to LPS in four AD brain specimens, which were positive when screened by immunofluorescence [199]. Bacterial endotoxins may be involved in the inflammatory and pathological processes associated with AD [200]. Indeed a number of studies indicate that the LPS-induced neuroinflammation can drive Aβ formation (e.g., [201–206]).

Interestingly, it has been observed that chronic infusion of the bacterial LPS into the fourth ventricle of rats reproduces many of the inflammatory and pathological features seen in the brain of AD patients [200, 201].

Previously we have reviewed the extensive published accounts suggesting a possible link between LPS presence and the pathological process of AD [34, 126, 207–211]. It is also well known that LPS presence is at least one of the causes of inflammation [212–214], and one of the hallmarks of inflammation is a hypercoagulable state [215–221]. Previously, we have seen changes in erythrocytes (RBCs), as well as hypercoagulation in the presence of LPS, where we added LPS to whole blood of healthy individuals or to platelet poor plasma [34]. We also reported on the presence of bacteria, which will indeed point to the presence of LPS, in whole blood of AD and Parkinson's disease patients, and also in fact inside RBCs [34]. We also discussed in detail the reasons why we might find bacteria in typically "sterile" blood, and suggested that these bacteria may be dormant (as operationally defined).

VITAMIN D, INFECTION, AND AD

While, in a sense, 'everything is connected to everything else', the role of the systems biologist is to highlight those metabolic networks and other processes whose variation (whether as a dependent or an independent variable – see [222]) are most pertinent to the outcomes of interest. Leaving aside the well-established roles of vitamin D in calcium and bone metabolism, it does seem to have a considerable impact on the immune system. To this end, there are some interesting clues (e.g., [223]) that link inflammation, infection, and vitamin D metabolism (and indeed elements of iron and vitamin D metabolism [224]), as well as AD [225–231]. Although the degree, and any mechanisms, of causality remain to

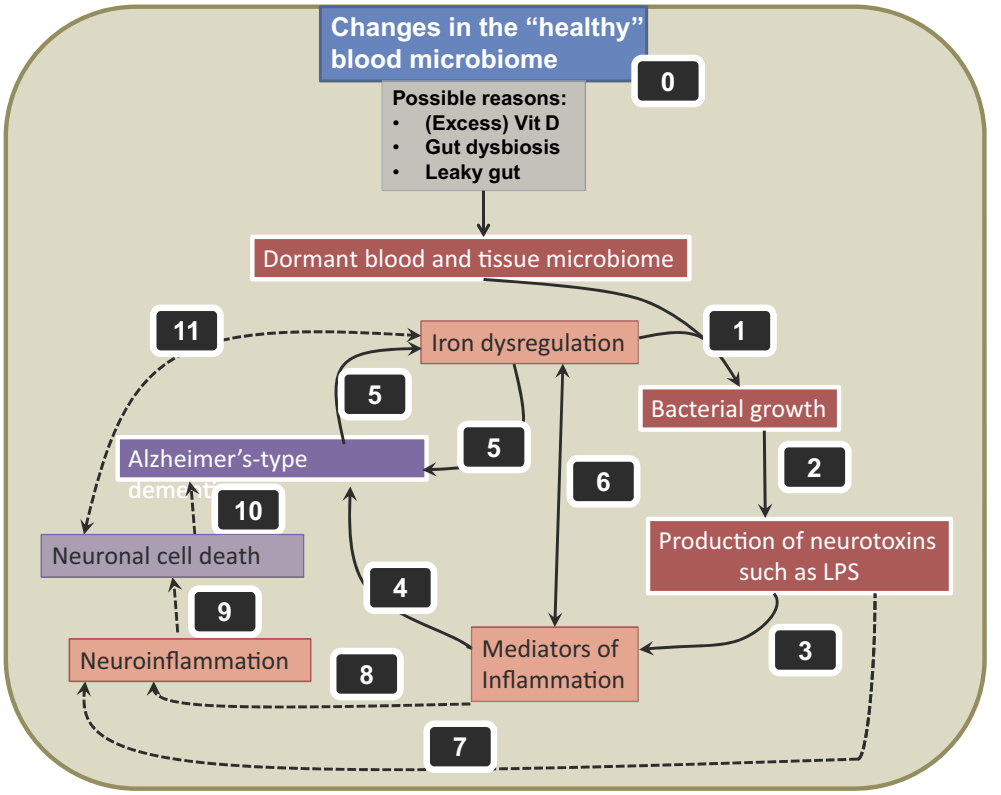

Fig. 3. A generalized systems scheme for microbial/iron-driven inflammatory disease in Alzheimer's-type dementia.

be seen, and the inter-relations are complex and non-linear [232, 233], there is an emerging consensus among a significant group of workers that chronic infection is intimately linked to detailed vitamin D status, and that this may provide a way in to useful therapies for a variety of chronic, inflammatory diseases (e.g., [101, 234–237]). The first issue concerns what in fact we mean by 'vitamin D'. Specifically, vitamin D may typically refer to two distinct forms: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), with some question as to whether D_2 is indeed useful as a vitamin supplement [238, 239]. The structures and metabolic products of vitamin $D_2/_3$ (of which only the hydroxy derivatives are in fact active, and the 1α,25-dihydroxyderivative especially) are given in Fig. 4.

In particular, Mangin and colleagues [235] have suggested that that low 25(OH)D is a consequence of chronic inflammation rather than the cause, and that tissue bacteria were responsible for an inflammatory disease process which results in high 1,25(OH)2D and low 25(OH)D (see also [237]). 1,25(OH)2D activates the vitamin D receptor (VDR) [240–244], a transcription factor that serves to induce the expression of over 900 genes, including for antimicrobial peptides [101, 223, 245–251] such as cathelicidin and beta defensins which attack (presumably non-dormant) pathogens [252]. In general, the innate immune system is enhanced and the adaptive immune system is inhibited by 1,25(OH)2D [235]. The general scheme, essentially as redrawn from [235], is given in Fig. 5. Other papers have also highlighted a relationship between low 25(OH)D and AD [226, 229, 230, 253, 254] and tend to imply that vitamin D supplementation should therefore be a solution. Obviously from a systems biology point of view, this does not follow directly, and there is evidence that the opposite can in fact be true [235, 236, 255]; clearly we need to know precisely the different roles of 25(OH)D and 1,25(OH)2D, and any effects on the CYP enzymes that produce them. More particularly, however, the complex, variable quality [256], and sometimes apparently contradictory, literature [257] is arguably better explained on the basis that there are separate populations who simply respond differently to vitamin D_3 supplementation [258–260]. Biomarkers (such as taurinuria; [261]) for genuine vitamin D deficiency may help disentangle this. Indeed, the

contradictory nature of any kinds of phenomena in which the 'same' additions are made to the 'same' system with very different results are typically explainable on the basis of uncontrolled variation. Thus the antioxidant ascorbate is actually pro-oxidant if unliganded iron is present [35]. Another explanation of such contradictions here involves the simultaneous presence of agonist and antagonist conformers of the VDR [262–264]. Finally, and in a different vein, the apoptotic versus proliferative effects of NF-κB are determined by the frequency rather than the amplitude of the NF-κB signaling molecule [43, 265, 266]. Vitamin D has significant effects on NF-κB [267–269]. Since there are also significant oscillations in ERK [270], VDR levels are partly dependent on ERK [271], and vitamin D3 also regulates circadian genes [272], these kinds of explanations based on the timing and frequency of oscillations (rather than simple metabolite concentrations) seem well worth exploring.

At all events, the nature(s) of the intracellular pathogens (and the cells in which they reside) is probably very wide, and at least one strategy for their persistence (in terms of their ability to evade the immune systems) is the adoption of cell-wall-deficient morphologies [148] or L-forms [273]. These, as well as more conventional structures, can of course be detected microscopically.

DIRECT DETECTION OF MORPHOLOGICAL CHANGES IN THE BLOOD OF AD PATIENTS

Pathologic RBCs and hypercoagulable fibrin(ogen) in AD patients

In previous work, we showed that the erythrocytes of AD patients were of highly anomalous shape, especially when serum ferritin levels were simultaneously raised [33] and that there was likely a hypercoagulable state (ascribed to the elevated LPS [34]). Here we now also show that AD RBCs are indeed abnormal, by using RBC and antibody-based fluorescent markers for spectrin (Ab11751) (red fluorescence) and Band-3 (Ab11012) (green fluorescence). Band3 is found in three distinct protein complexes associated with the erythrocyte membrane: an ankyrin-dependent tetrameric band3 complex, a dimeric band3 complex bound to the protein 4.1-glycophorin C junctional complex, and freely diffusing dimeric band3 complexes [274, 275]. Band 3 can also bind to spectrins, the internal scaffold for erythrocyte shape, via ankyrin, suggesting that band 3 contributes to the membrane-cytoskeleton interactions that help to define erythrocyte shape and stability [276, 277]. Structural alterations to the phospholipids, as well as band 3 and spectrin, cause RBC physical shape changes, which can be detrimental to their normal functioning [97, 278]. Under normal conditions, the neutral phospholipids, phosphatidylcholine, and sphingomyelin are mostly found on the outside, and the charged phospatidylserine (PS), phosphatidylinosirol, and phosphatidylethalolamine, are found mostly on the inner membrane leaflet. However, during inflammation, the erythrocyte membrane leaflet phospholipids becomes more symmetric as PS is externalized, resulting in RBC membrane vesicle formation and ultimately microparticle shedding, with subsequent pathological shape changes of RBCs [279]. PS is normally found only on the intracellular leaflet of the plasma membrane in healthy cells, but during early eryptosis (RBC programmed cell death) [280–284], membrane asymmetry is lost and PS translocates to the external leaflet [285]. For a detailed review on the role of the RBC membrane and changes therein due to inflammation, see [286].

Figure 6A shows a typical example of confocal microscopy of a healthy RBC and Fig. 6B shows a typical scanning electron microscopy (SEM) image of a representative RBC from an age-controlled healthy individual, while Fig. 6C and D show confocal and SEM images of a representative sample from an AD individual. Figure 6A shows intense green fluorescence on the rim of the RBCs and less intense toward the inside of the RBC. There is little to no red fluorescence specifically on the rim of the RBCs indicating the presence of the spectrin. Where there is some red staining, it is more toward the inside of the RBCs and much less intense than the green band3. In the RBCs of the AD individuals (Fig. 6C), the red fluorescence is much more visible, and the red fluorescence is found not only on the inside of the RBCs but also on the rim and outside of these cells unlike the control group. This suggests a structural membrane disorder, typically associated with eryptosis, which is often enhanced by cytoplamic calcium activity and also characterized by cell membrane scrambling and cell shrinkage [287, 288]. Particularly the disarrangement of spectrin and band 3 positional changes are two important markers to determine structural damage to the membrane that will result in changes to elasticity and pliability of RBCs [286]. SEM images comparing healthy and AD RBC ultrastructure, clearly show that the RBCs from

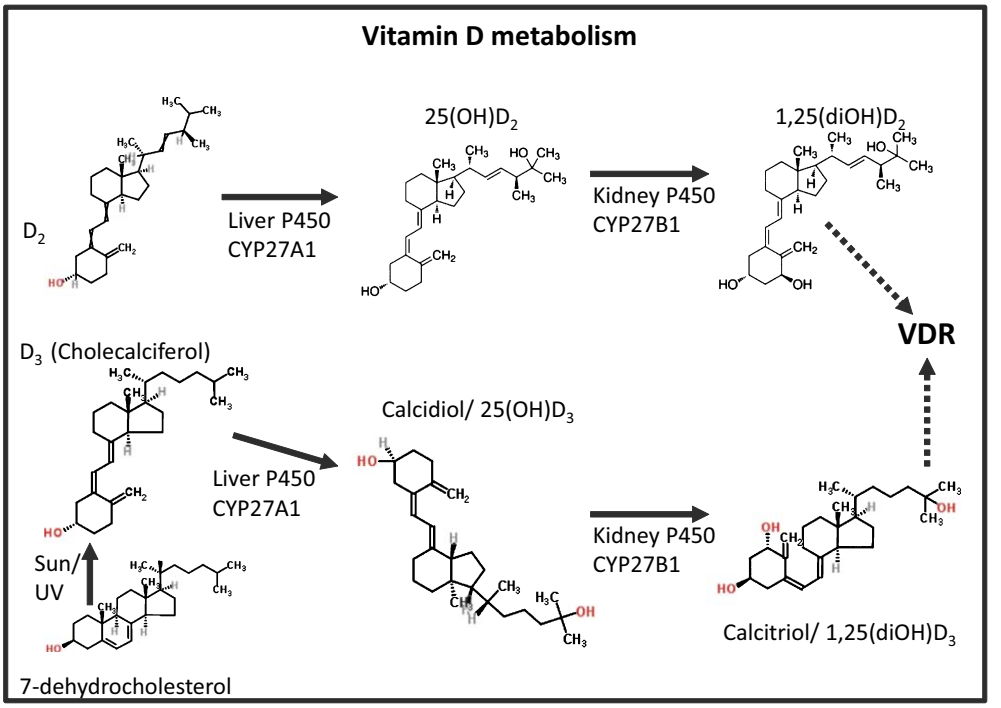

Fig. 4. The structures and major metabolic products of vitamin $D_2/_3$. The dihydrohylated derivatives are by far the most active in terms of binding to the vitamin D receptor.

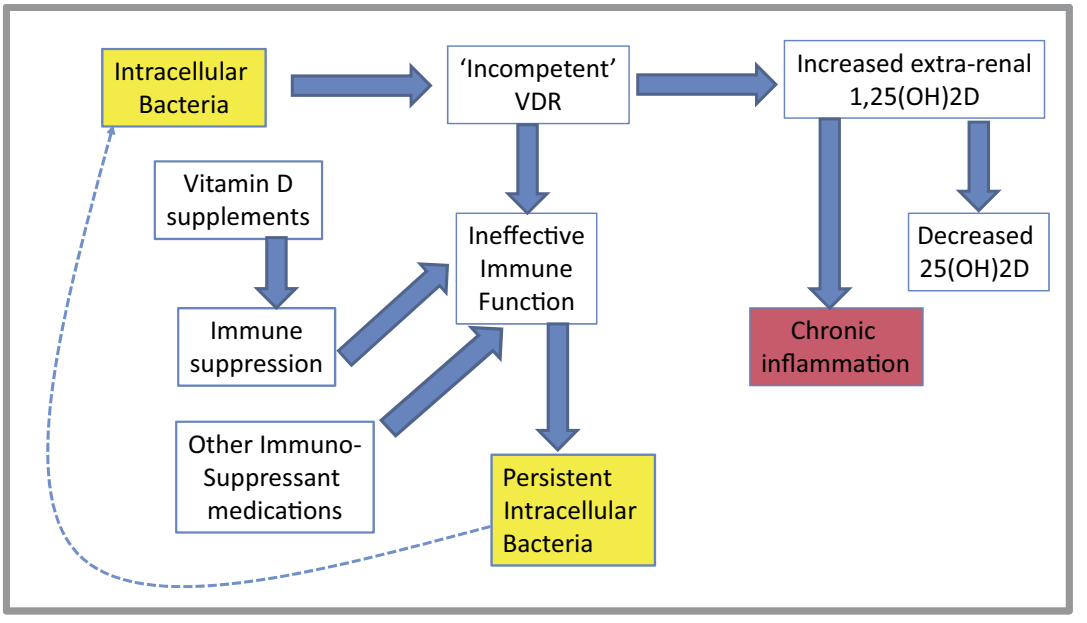

Fig. 5. A general scheme of some of the roles of vitamin D and its metabolites in chronic infection: (essentially as redrawn from [235]).

AD individuals have an eryptotic structure. Eryptosis is visible in most of the RBCs from AD patients, and also in those with Parkinson's disease [95]. Additionally to the eryptotic structure of the RBCs, bacteria were also visible with SEM in the same AD sample (Fig. 6E, F).

As well as changes in AD RBCs, we previously found that pathologic fibrin fiber formation

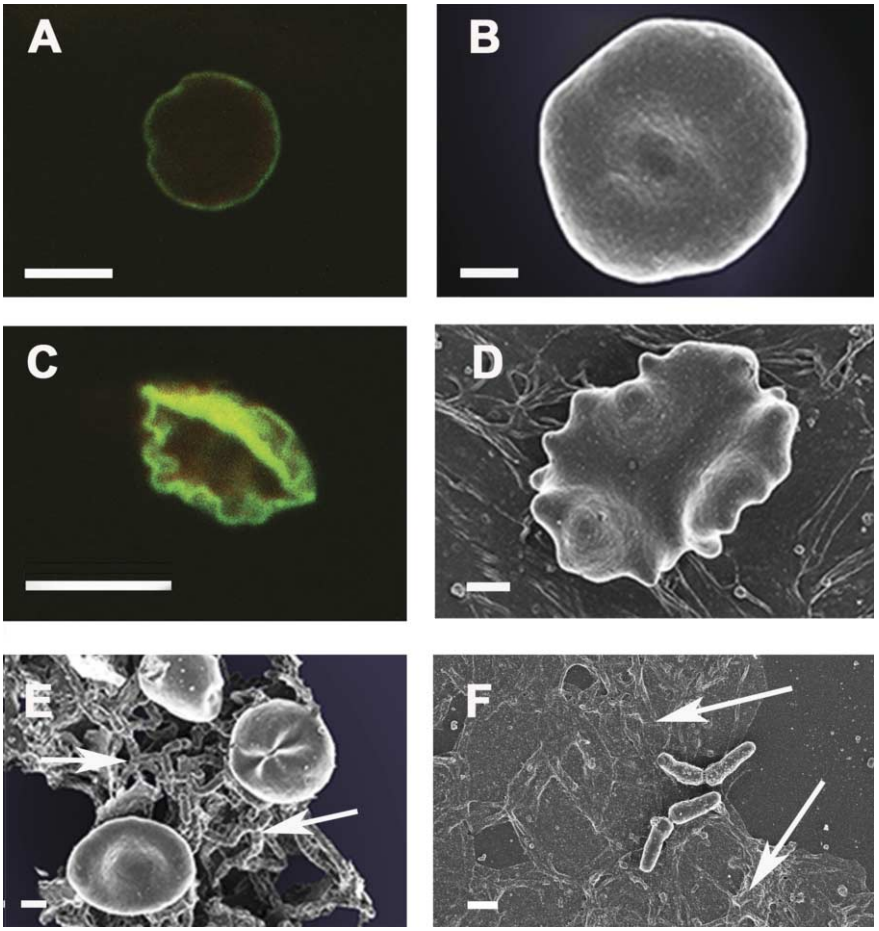

Fig. 6. Confocal and scanning electron microcopy (SEM) of health and Alzheimer's-type dementia RBCs. The fluorescent markers spectrin (Ab11751) (red fluorescence) and Band-3 (Ab11012) (green fluorescence) were used in confocal microscopy. A) Confocal micrograph of a healthy RBC; B) SEM micrograph of a healthy RBC; C) Confocal micrograph of an Alzheimer's-type dementia RBC; D) SEM micrograph of an Alzheimer's-type dementia RBC; E) SEM micrograph showing bacteria between RBCs; and F) of bacteria and matted fibrin. Scale bar of SEM micrographs: 1 μm; and for confocal: 5 μm.

(associated with hypercoaguation) is also present in AD, and may therefore be used as a further and useful inflammatory indicator [34]. As seen in pathological changes in RBCs, oxidative damage, increased iron levels, and inflammation are also all reasons for the development of hypercoagulability [95–97, 289–293]. Hypercoagulability is closely associated with increased fibrin(ogen) in AD patients, while hypercoagulation has been observed in blood vessels positive for amyloid in mouse and human AD samples [294]. A changed fibrinogen structure has been implicated in the development of neuroinflammation [295, 296], and memory deficits and increased fibrinogen levels in AD are noted to be a strong indicator of cerebrovascular risk, as fibrinogen specifically binds to Aβ, thereby altering fibrin clot structure and delaying clot degradation [297]. In a previous paper, we looked at the viscoelastic and ultrastructural properties of AD plasma and whole blood by using scanning electron microscopy, thromboelastography (TEG®) and the Global Thrombosis Test (GTT®) [34]. TEG® analysis showed a hypercoagulable state in AD, while TEG® results, where LPS was added to uncitrated blood, showed the same trends as were found with the AD patients. The GTT® results (where only platelet activity is measured) were not affected by the added LPS, suggesting that LPS does not directly impact platelet function [34]. See Fig. 7 for an ultrastructural comparison of platelet poor plasma smears (treated with thrombin) from a healthy (age-controlled) individual and from an AD individual.

Although pathophysiological changes in RBCs and fibrin fiber structure are not unique to AD, they are hallmarks of systemic inflammation [96], and as

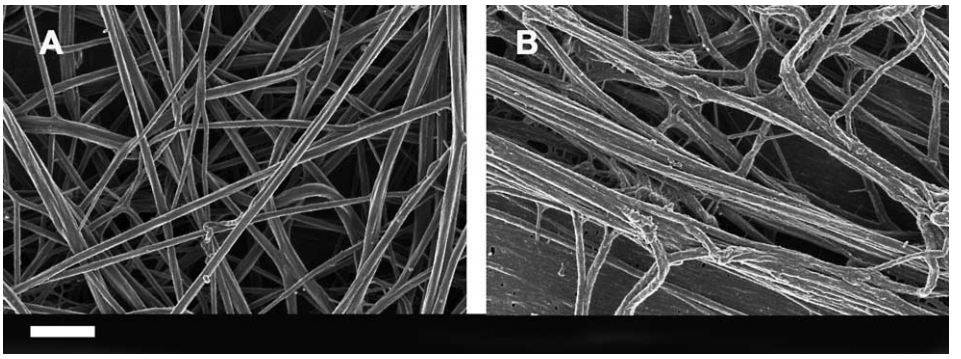

Fig. 7. Platelet poor plasma of A) healthy (age-controlled) individual; and B) an Alzheimer's-type dementia individual. Thrombin (20 U.mL^{-1}) was added at a final concentration of 57.7 nM. Scale bar: 1 μm.

noted here LPS may play a role in the biochemical pathways that may destabilize RBC and fibrin structure. As RBCs are extremely vulnerable in the presence of pro-inflammatory molecules, hydroxyl radicals, oxidative stress, and LPS, they may possibly be used as a 'healthiness' indicator of AD patients. Currently we have few actual markers of AD status, and we note that the latest NIH guidelines suggest that clinical medicine should focus on precision medicine [298] and that individualized medicine should in the future, form an essential part in the diagnosis and treatment of patients. We therefore suggest that RBC and fibrin morphology could be used as "health indicators". Here we do not of course suggest that they should be used as diagnostic tools for AD per se, but rather as a healthiness indicator of the overall systemic inflammatory status of patients after diagnoses.

CONCLUSION

Modern molecular biology had become a little obsessed with a presumed need for hypotheses, and it has taken the post-genomic era to remind scientists of the virtues of scientific induction and data-driven biology [299, 300], often intertwined with a systems biology approach. A typically nice example is a hypothesis-free discovery biology paper [301] in which the authors sought to identify those pathways that were most intimately involved in the development of prion disease. Genes involved in iron metabolism were among the most highly involved [301].

In a similar vein, we have brought together the evidence underpinning a coherent and self-consistent view of the linked contributions to AD progression of iron dysregulation, the resuscitation of dormant bacteria, and the shedding of the highly inflammatory LPS that can induce both cytokines and apoptosis (see Figs. 2 and 3). As with any systems approach, it implies the need for pharmacological interventions at multiple points (e.g., [302–305]). The role of the systems pharmacologist, based on knowledge of the most important pathways proposed herein, is to develop them.

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Iron Withholding: A Defense Against Disease

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Abstract. Excessive and misplaced iron promotes an array of neurodegenerative and endocrine diseases as well as cardiomyopathy, arthropathy, neoplasia and infection. Vertebrates maintain an iron withholding defense system designed to prevent accumulation of redox-active (free) iron in sensitive sites and to sequester the metal in innocuous packages. Numerous genetic, behavioral and environmental factors counteract the defense system. Our increasing awareness of the pathologic roles of iron, as well as of the methods for prevention of iron loading coupled with intensified research and development of tissue specific iron chelator drugs, can be expected to yield marked improvements in human health.

Keywords: Alzheimer's disease, bacteria, cardiovascular disease, cerebrovascular disease, dementia, infectious disease, iron, iron withholding defense, neoplastic disease, neurodegenerative disorders

IRON WITHHOLDING

"The host plays an active part in the depletion of utilizable iron." [66]

In order to safely transport and employ iron, cells must prevent over accumulation of the metal in the redox-active (free) state. The latter is toxic in several ways. The attributes of iron that provide diversified metabolic utility likewise render the metal hazardous for iron-dependent cells [74]. Iron catalyzes generation of hydroxyl radicals which intensify oxidative stress. Consequences include enhancement of radiosensitivity, mutation, lipid peroxidation, polysaccharide depolymerization, enzyme inactivation, degenerative aging and cell death. The metal also is hazardous to hosts by serving as a growth-essential nutrient for invading microbial and neoplastic cells [127].

With the exception of a few bacterial species that use manganese, cells of all other forms of life are iron dependent. Thus vertebrates, invertebrates, plants and nearly all prokaryotes possess systems that attempt to control iron quantity and to withhold excess amounts from sensitive intracellular organelles. An overview of the iron withholding defense system is contained in Table 1 [93,137].

Especially important in lowering redox-active iron levels during the inflammatory defense process are such acute phase reactants as hepcidin, ferritin and lactoferrin. Very early in the process, activated macrophages secrete IL-6 which induces hepatocytes to form hepcidin. This 25 amino acid cysteine-rich hormone binds to ferroportin; the complex then is inactivated in lysosomes [97]. Thus the normal ferroportin-induced iron recycling by macrophages is dampened and plasma iron level is markedly reduced.

To safely contain the intra-macrophage iron surge, synthesis of heavy chain ferritin promptly is activated by TNFα in an NF-kβ-dependent manner [96]. The ferroxidase of H ferritin converts Fe(II) to Fe(III) as iron is being internalized and sequestered in the ferritin mineral core.

Migration of polymorphonuclear neutrophils to the inflammatory site, followed by their degranulation, releases lactoferrin. The pH value at the site tends to be lowered by catabolic metabolism of the host defense

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Table 1
Iron withholding defense system

Constitutive components
Siderophilins
Transferrin in plasma, lymph, cerebrospinal fluid
Lactoferrin in secretions of lachrymal & mammary glands and of respiratory, gastrointestinal & genital tracts
Ferritin within host cells
Processes induced at time of invasion or trauma
I. *Prompt reduction of 80% in dietary iron absorption and 70% reduction in plasma iron*
Increased hepatic synthesis of hepcidin (inactivator of ferroportin) to suppress duodenal iron absorption & release of recycled macrophage iron into plasma
Macrophage enhancement of DMT-1 expression and inhibition of ferroportin synthesis to withhold iron from invaders
Increased synthesis of ferritin to safely sequester withheld iron
II. *Removal of iron from sites of invasion*
Release of neutrophils from bone marrow into circulation and then into diseased sites
Release of apolactoferrin from neutrophil granules followed by binding of iron in diseased sites
Macrophage scavenging of ferrated lactoferrin in diseased sites
Hepatic release of haptoglobin and hemopexin (to bind extracellular hemoglobin and hemin, respectively)
III. *Suppression of microbial iron metabolism*
Macrophage synthesis & secretion of siderocalin which captures microbial siderophores
Macrophage synthesis of nitric oxide (from 1-arginine) which depresses TfR expression and disrupts invader iron metabolism
Suppression of intra-macrophage microbial cell growth via enhanced synthesis of Nrampl by the host cells
IV. *Induction in B lymphocytes of synthesis of immunoglobulins to iron-repressible cell surface proteins* that bind either heme, ferrated siderophilins or ferrated siderophores

Table 2
Some conditions that compromise the iron withholding defense system

Genetic disorders
Aceruloplasminemia, African siderosis, hemochromatosis, transfusion dependent: myelodysplasia, sicklemia, thalassemia
Behavioral factors
– *Ingestion* of excessive amounts of: heme (red meat), iron supplements, ascorbic acid, ethanol, food that has been adulterated with iron
– *Inhalation* of iron-containing items: asbestos, coal, ferriferous ores & metals, tobacco smoke urban & subway air particulates
– *Injection* of excessive amounts: iron saccharates, whole blood, erythrocytes
Pathological conditions
Release of body iron into plasma: efflux of erythrocyte iron in hemolytic conditions
efflux of hepatocyte iron in hepatitis, loss of spleen
myelo-ablative conditioning prior to cell/tissue transplant

cells as well as by any invading microbial or neoplastic cells. Fortunately, among the known siderophilins, lactoferrin uniquely scavenges iron at pH values as low as 3.5 [140].

In the healthy state, there should never be an over-accumulation of free iron. Unfortunately, although humans have an intricate mechanism for controlling intestinal absorption of iron, they lack a mechanism (other than bleeding) for elimination of grossly excessive quantities. The manifold ways in which acquired iron exceeds physiologically appropriate needs are summarized in Table 2. For the past sixty years, some merchandisers of processed foods have claimed that "iron-fortified" foods will make us healthier and stronger. Unhappily, this is true only for the small minority of persons who truly are iron deficient.

In developed countries, accumulation of excess iron in males can begin in early adulthood and then increase almost linearly with age. Females delay over-accumulation by menstruation and/or pregnancy. Post-menopausal women can attain parity with men in iron burden within a few decades. As humans acquire the perilous metal, they are forced to contain it (in ferritin/hemosiderin) within cells in a great variety of tissues. These include, but are not limited to, brain, heart, liver, pancreas, pituitary, joints, bone, lung, spleen and skin.

Organ distribution of contained iron differs widely among individuals. Moreover, the amount of tissue

Table 3
Diseases for which excessive/misplaced iron can be a risk factor*

Cardiovascular	*Obstetric*
atherosclerosis	neonatal hemochromatosis
cardiomyopathy	pre-eclampsia
hypertension	*Oncologic*
ischemic stroke	breast cancer
venous leg ulcer	colorectal cancer
Dermatologic	hepatic carcinoma
porphyria cutanea tarda	Kaposi sarcoma
Endocrine	leukemia
diabetes	lung cancer
endometriosis	*Ophthalmic*
growth deficiency	macular degeneration
hypogonadism	*Orthopedic*
hypothyroidism	gout
Hepatic	hemophilic synovitis
cirrhosis	osteoarthritis
steatohepatitis	osteoporosis
viral hepatitis	*Otologic*
Infectious	aminoglycoside toxicity
bacterial	*Pediatric*
fungal & protozoan	Down syndrome
Neurologic and neurodegenerative	epilepsy
Alzheimer's disease	sudden infant death syndrome
Huntington' disease	*Pulmonary*
multiple sclerosis	cystic fibrosis
Parkinson's disease	ozone lung injury
pantothenate kinase	pneumoconiosis
prion disease	*Renal*
amyotrophic lateral sclerosis	aminoglycoside & vancomycin toxicity
depression	
Friedreich's ataxia	
cerebrovascular disease	

*Modified from Table 28 [139].

damage varies not only with iron quantity but also with the specific tissue. For instance, iron kills anterior pituitary cells at 1.2 μM whereas hepatic cells resist destruction at levels 10-100 times greater [33].

During the past several decades, a considerable profusion of diseases have been recognized to be associated with iron mismanagement (Table 3). Provisonally, the deleterious action of iron can be assigned to one of five categories (Table 4). As further clinical and laboratory research becomes available, some of the assignments will require adjustment.

Following are brief summaries of selected items of current interest on toxic iron in five groups of diseases: neurologic, cardio- and cerebro-vascular, endocrine, oncologic and infectious. The review concludes with a section on prophylactic and therapeutic measures to aid iron withholding.

IRON ACCUMULATION AND ALZHEIMER'S DISEASE

"The underlying pathogenic event in oxidative stress is cellular iron mismanagement." [122]

Rapidly accumulating data show that, similarly to inflammation, an involvement of iron and iron-mediated oxidative stress is a common denominator of various neurodegenerative and chronic neuropsychiatric disorders, including Alzheimer's disease (AD) which is the most frequent cause of dementia. Iron is important for brain oxygen transport, electron transfer, neurotransmitter synthesis, and myelin production [120]. Iron homeostasis in the brain is not only important for maintaining normal brain function but also for the prevention of diseases. Redox active brain iron accumulation in aging [72] and in various chronic neurodegenerative and neuropsychiatric disorders [8,64, 79,84,116,151] is well documented. In addition to AD [16,65,78,112,151], increased iron was reported to occur in Down syndrome [40], Parkinson's disease (PD) [7,8,49,64,122], diffuse Lewy body disease (DLBD), amyotrophic lateral sclerosis (ALS) [126], multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal ganglionic degenera-

Table 4
Examples of action of iron in specific diseases*

I.	*Iron, by itself can initiate the disease*
	cardiomyopathy [91], growth deficiency [107], hypogonadism [9], hypothyroidism [34], hemophilic synovitis [58], lung cancer [132], osteoporosis [138], pneumoconiosis [149]
II.	*Iron can be a cofactor in promoting the disease*
	Alzheimer's disease [16,78,151], atherosclerosis [62], bacterial infections [129], diabetes [32], endometriosis [26], fungal & protozoan infections [130], gout [50], multiple sclerosis [59], osteoarthritis [110], oto-toxicity [45], ozone lung injury [51], renal toxicity [2,86]
III.	*Iron deposits are observed in disease-associated tissue sites*
	basal ganglia in pantothenate kinase neurodegeneration [55], brain in prion disease [4], hepatocytes in cirrhosis [90], hepatocytes in steato- and viral- hepatitis [30,39], macula in macular degeneration [57], microglia in Huntington's disease [113], mitochondria in Friedreich's ataxia [104], pulmonary secretions in cystic fibrosis [102], soft tissue in Kaposi's sarcoma [114], substantia nigra in Parkinson's disease [122]
IV.	*Body iron loading is associated with above-normal incidence of disease*
	amyotrophic lateral sclerosis [126] breast cancer [61], colorectal cancer [89,105], hepatic carcinoma [85] depression [41] Down syndrome [40] epilepsy [60], hypertension [99] inflammatory bowel disease [71,92] ischemic stroke [37], leukemia [31], pre-eclampsia [101], venous leg ulcer [148], porphyria cutanea tarda [13], sudden infant death syndrome [134]
V.	*Maternal antibodies can impair fetal iron metabolism*
	fetal or neonatal death in neonatal hemochromatosis [143]

*Modified from Table 2 [139].

tion (CBD), ALS/parkinsonism dementia complex of Guam (ALS/PDCG), Huntington's disease (HD) [64, 113], prion diseases [4,14], multiple sclerosis (MS) [59, 116], mood disorders [41], epilepsy [60], aceruloplasminemia, hereditary ferritinopathies [143], panthotenate kinase-associated neurodegeneration type 2 (PKAN) [55], Friedreich ataxia [104], cardiovascular and cerebrovascular diseases [37,62,99] and macular degeneration [57]. An excess of iron generates free radicals and damages cells [68,116], accordingly it is apparent that oxidative stress is intimately involved in the pathogenesis of these disorders. Increased iron in AD in association with senile plaques was first described by Goodman in 1953 [54]. His observation was repeatedly reinforced by others and validated by the use of various specific and sensitive techniques [10,20,21, 23]. Oxidative stress is one of the earliest events in AD and seems to be involved in the onset, progression and pathogenesis of the disease [16,151]. Deregulation in brain iron metabolism is multifactorial and comprises nongenetic and genetic factors. It might occur at multiple levels, including iron uptake and release, storage, intracellular metabolism and regulation [63,151] Iron levels are regulated within cells by iron regulatory proteins (IRPs). IRPs by binding to iron responsive elements (IREs) of several genes encode key proteins such as the transferrin receptor (TfR) and ferritin. Transferrin is involved in the physiological transport and utilization of iron. Transferrin concentration is decreased in AD and in other neurodegenerative disorders. Concurrently, the hypoxia-inducible factor (HIF) has also been shown in previous studies to regulate intracellular iron by binding to HIF-responsive elements (HREs) that are located within the genes of iron-related proteins such as TfR and heme oxygenase-1 (HO-1) [68]. Disruption in brain iron homeostasis through alterations of iron regulatory proteins can increase the vulnerability of cells to oxidative stress [23,98]. Changes in superoxide levels due to alteration of superoxide dismutase (SOD) activity also affect iron metabolism in glial and neuronal cells [24,25]. Lactoferrin (LF) which is secreted by ectodermal tissues is similar in structure to transferrin and plays a role in natural defense mechanisms in mammals. It is upregulated in neurodegenerative disorders. Lactoferrin exerts an anti-inflammatory function via its inhibitory effect on hydroxyl radical formation and, by its antioxydative properties prevents DNA damage [108]. The combination of the C2 variant of the transferrin gene (TF-C2) and the C2 82Y allele of the haemochromatosis (HFE C282Y) gene, or the combination of HFE C282Y and HFE H63D are risk factors for developing AD. Carriers of such combination were at 5 times greater risk for AD. Additional apolipoprotein E epsilon4 (APOE4) allele further increases the risk of AD [22,70,87,106]. Oxidative damage, produced by mutant Cu/Zn superoxide dismutase (SOD1), and an increased frequency of H63D mutation was also reported to occur in ALS [69,147]. Brain ferritin iron may also influence age- and gender-related risk of neurodegeneration [3].

Elevated levels of combinations of cholesterol and iron have been observed to promote AD in animals [52] and to be a risk factor in humans [76]. In a set of 6,558 US adults, followed from 1974 to 1992, an elevated risk of AD occurred if both cholesterol and iron were above normal (Table 5). There is increasing evidence to support a role for both the amyloid-β protein precursor (AβPP) and its proteolytic fragment, amyloid-β

Table 5
Association of cholesterol and iron with development of Alzheimer's disease

Cholesterol mg/dL*	Tf iron saturation%*	Alzheimer's disease %**
< 261	< 34.9	1.00
> 261	< 34.9	1.60
< 261	> 34.9	1.35
> 261	> 34.9	3.00

*Values obtained at baseline for 75th percentile.
**Development of Alzheimer's disease within 18 years after baseline.
Data from figure 1 [76].

peptide (Aβ) in metal ion homeostasis. Iron participates in the aggregation of Aβ and may play a role in neurofibrillary tangle formation, tau phosphorylation and in secretase cleavage of AβPP [1,35,38,103]. It was suggested that transferrin limits fibrillar formation and cytotoxicity of Aβ [53]. Inflammatory processes play a key role in the pathogenesis of AD and several other neurodegenerative and neuropsychiatric disorders including cerebrovascular disorders. Compelling evidences exist that iron is involved in inflammatory reactions, therefore, it is not surprising that both inflammation and iron accumulation are common denominators of these various chronic disorders [150]. *In vivo* magnetic resonance imaging of acute brain inflammation using microparticles of iron oxide was recently reported [80].

Therapeutic strategies derived from application of iron chelators and new drugs diminishing iron accumulation and oxidative stress is promising and warrant further investigational effort in AD and other neurodegenerative and neuropsychiatric disorders [8,103].

CARDIO- AND CEREBROVASCULAR DISEASES

"It is now quite apparent that excessive iron in either arteries or heart muscle cells is detrimental to a properly functioning cardiovascular system." [136]

Cardiomyocytes are highly susceptible to iron loading. In thalassemia patients who receive monthly blood transfusions but inadequate iron chelation, cardiomyopathy is the leading cause of early death [146]. Iron deposits are associated with cardiac hypertrophy and dilation as well as with degeneration of myocardial fibers. In iron loaded gerbils, the metal is contained in high amount in left ventricular cells and in the epicardium [91].

A link between iron loading and atherosclerosis has been observed often in animal models [117]. For instance, in rabbits fed a 1% cholesterol diet, a seven-fold increase in iron concentration had occurred in arterial tissue by the onset of lesion formation. Iron reduction by bleeding or chelation suppressed lesion development [100].

Human arteries, likewise, develop atherosclerosis in association with iron. Arterial lesions have been reported to contain 3–17 times more iron than healthy controls [115]. Iron loaded macrophages support growth of such intracellular bacteria as Chlamydia [118] and Coxiella [135] that have been linked with the chronic inflammatory process of atherosclerosis.

Additionally, oxidative stress of lipids induced by iron may play a role in artery damage. In a sample of 13,932 US adults, elevated C-reactive protein was associated with increased ferritin plus high LDL or low HDL [77]. In a study of 38 atherosclerotic patients, the level of low molecular mass iron in plaques removed in endoarterectomy was directly correlated with body iron loading and the severity of the disease [67].

In 9,178 persons followed for 24 years, 504 developed ischemic cerebrovascular disease of whom 393 had ischemic stroke [37]. In those who were H63D homozygotes, the incidence of stroke was increased between two and three fold. No increase occurred in persons with the C282Y mutation. Indeed, hemochromatotic persons who are homozygous for the C282Y mutation appear also to be protected from developing atherosclerosis. Because this mutation results in lack of hepcidin, their macrophages are very low in iron [141].

Ferritin levels of 134 consecutive acute stroke patients treated with i.v. tissue plasminogen were tested for ferritin at the time of treatment. Patients whose ferritin was greater than 79 ng/ml had increased risk of poor clinical outcome, hemorrhagic transformation and brain edema [83]. In a study of 38 men with essential hypertension and of 40 healthy controls, 21% of the former and none of the latter were hyperferritinemic [99]. Elevated iron was associated also with insulin resistance.

ENDOCRINE DISEASES

"Despite its frequency and effect on the endocrine system, haemochromatosis has attracted surprisingly little attention in endocrinology and fertility textbooks." [123]

A majority of humans who develop iron loading, irrespective of the cause, proceed to have one or more impaired endocrine glands. Especially sensitive to iron toxicity are cells of the anterior pituitary. When iron loading occurs early in life, as in thalassemia, a deficiency of growth hormone results in short stature. When excessive iron occurs in older individuals, suppression of gonadotrophic hormones causes impotence in males and amenorrhea, loss of fertility and early menopause in females [9,29].

Furthermore, the metal destroys pancreatic insulin-forming beta cells. Thus glucose intolerance often develops. Noted especially is a marked increase in patients with type 2 diabetes [42,119]. At least one-third of patients with type 2 diabetes have elevated serum ferritin [145]. Reduction in insulin secretion often is accompanied by increased insulin resistance. In hereditary hemochromatosis, up to 60% of untreated patients may develop type 2 diabetes. Lowering of iron load either by phlebotomy or chelation can result in a significant reduction in Hgb A1C and improvement or reversal of the diabetic condition [145].

Late onset of type 1 diabetes likewise is associated with iron loading. In a group of 716 adults who had onset after 30 yrs, those homozygous for the C282Y gene mutation were 4.6 times more likely to have the disease than were normal persons [36].

In a group of thalassemic teen-aged children, 8.5% had impaired glucose tolerance and 20% diabetes [17]. In a prospective study of 1,038 randomly selected individuals, those in the highest quartile of body iron were 2.4 times more likely to become diabetic within the next four years [145].

As in the pituitary and pancreas, iron loading occurs also in the thyroid gland. Accumulation of iron up to 25 times normal has been observed in the thyroid in untreated hemochromatotic patients [34]. Damage to the parathyroid gland also has been noted [95].

ONCOLOGIC DISEASES

"One might worry about the iron injectable compounds which are being tested and used. One could almost guess that someone is going to find iron dextran carcinogenic," [46]

Iron is carcinogenic as a mutagen, as an inhibitor of the tumorcidal action of macrophages, and as an essential nutrient for growth of cancer cells [128]. In both animals and humans, primary neoplasms develop at body sites of excessive iron deposits. Inhaled iron is associated with respiratory tract cancers [132], ingested iron with colorectal malignancies [15,89], skin-exposed iron with sarcomas [114], and whole body iron loading with hepatomas [128].

Body iron that is increased because of gene mutations involved in iron metabolism has been linked to a variety of neoplasms. In patients with multiple myeloma ($n = 92$), breast cancer ($n = 165$) and colorectal cancer ($n = 173$), the odds ratio for carriers of the C282Y mutation as compared with the wild type was 2.0. The odds ratio was increased to 7.17 in C282Y carriers who also were homozygous for a transferrin receptor mutation at serine 142 [5]. Other studies have reported associations of hemochromatosis mutations with breast cancer [56, 61] and with colorectal cancer [105,111]. In a set of 27 patients with acute lymphoblastic leukemia (ALL), 44% carried the H63D mutation whereas in normal controls, the frequency was 25% ($P = 0.02$) [124]. In that study, no difference was observed between controls and patients with acute myeloid or acute premyelocytic leukemia.

Tissue iron that is increased because of behavioral factors similarly has been linked to a variety of neoplasms. Numerous studies have reported that workers in ferriferous industries have an elevated risk of respiratory tract cancers [132]. Likewise, persons who inhale varieties of asbestos that are comprised of iron (but not magnesium) silicates are at high risk for development of lung cancer and of mesothelioma [132]. Moreover, the risk of lung cancer in persons who inhale iron-contaminated tobacco smoke is well documented [132].

Dietary behavior also is important in accumulation of excessive body iron. Of special concern is the readily absorbable heme iron content of red meat. In a study of 90,659 premenopausal women, 1,021 developed invasive breast carcinoma [18]. Greater red meat intake strongly was related to elevated risk of estrogen and progesterone receptor-positive breast cancer but not to cancers that were estrogen and progesterone negative.

World wide areas of high incidence of Kaposi sarcoma are characterized by a substrate of fertile reddish-brown volcanic clay soil [114]. After aluminum, the

Table 6
Microbial genera with strains whose growth in body fluids, cells, or intact vertebrate hosts is stimulated by misplaced or excess iron

Fungi: Aspergillus, Candida, Cryptococcus, Histoplasma, Paracoccidioides, Pneumocystis, Pythium, Rhizopus, Trichosporon
Protozoa: Entamoeba, Leishmania, Naegleria, Plasmodium, Toxoplasma, Trichomonas, Tritrichomonas, Trypanosoma
Gram positive & acid fast bacteria: Bacillus, Clostridium, Corynebacterium, Erysipelothrix, Listeria, Mycobacterium, Staphylococcus
Gram negative bacteria: Actinobacter, Aeromonas, Alcaligenes, Campylobacter, Escherichia, Helicobacter, Klebsiella, Legionella, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia

*Modified from Table 1 [131].

most abundant mineral in the clay is iron. Barefoot peasants acquire ultra-fine soil particles through the skin of their soles. In Africa, persons with both the environmental skin invasion plus a genetic tendency to iron loading (African siderosis) are especially at risk.

INFECTIOUS DISEASES

"Of the myriad of competitive interactions known to occur between host and colonizing or infecting microbes, the struggle for micronutritional iron is among the most prominent." [88]

Bacterial fungal and protozoan pathogens (Table 6) have one or more strategies for securing host iron. These include (1) cell surface binding of ferrated transferrin or lactoferrin and extraction and assimilation of the metal; (2) synthesis of low molecular mass siderophores that extract the metal from transferrin with subsequent binding and uptake of the ferrated siderophore or of the metal; (3) lysis of erythrocytes, digestion of hemoglobin, and binding and assimilation of heme; and (4) assimilation of host intracellular iron derived from pools of low molecular mass iron binding compounds [94,129,131].

Successful pathogens often employ differing strategies depending on the particular biochemical environment. Flexibility especially is important for microbial strains that, at various times, live in different tissues of the host, in different hosts, or outside of hosts. For example, *Helicobacter pylori* obtains iron directly from lactoferrin when growing in the gastric lining but uses heme when it invades the gastric wall.

Some potential pathogens are sufficiently impaired in iron acquisition ability so as to be dangerous mainly in hosts with such iron loading conditions as African siderosis, β-thalassemia, or hemochromatosis [133]. However, even in iron-normal hosts, increased risk of infection can be acquired simply by over-ingestion of iron [47,109,121], over-inhalation of iron [132], or over-injection of iron [121].

The important role of iron in infection and the risk of infection following redox active iron accumulation are both well established [94,133,134]. It is noteworthy that dementia can be caused by chronic bacterial infection resulting in a slowly progressive cognitive decline which may occur decades following the primary infection. One characteristic lesion of this syphilitic paretic dementia (general paresis) caused by the spirochete Treponema pallidum is the accumulation of iron in the brain. Fast detection of the so called "paralytic iron", at the time of autopsy on macroscopic brain samples, was used as diagnostic tool for syphilitic infection [81].

PREVENTION AND THERAPY OF IRON LOADING

"... unavailability of meat or prolonged and heavy use of tea leaves, which eventually led to development of iron deficiency, may result in better survival in epidemics." [27]

In a cohort of 1401 US adults, 67–96 yrs, 70% had ferritin level >60 ng/ml whereas only 2.7% were iron deficient and only 1.2% had iron deficiency anemia [44]. Elevated iron was significantly associated with consumption of (1) non-heme iron supplements, (2) red meats (high in heme iron), and (3) fruit (high in ascorbic acid, an enhancer of non-heme iron absorption). In contrast, consumption of whole grains (high in phytates that inhibit iron absorption) was inversely correlated with elevated iron.

In a 12 yr study of 9,229 persons, 35-70 yrs at baseline, persons who had elevated transferrin saturation and who reported high dietary iron or high meat consumption had a three-fold increased risk of dying within the study period [75].

The phenolic iron chelating natural products in green and black teas have a strong affinity for non-heme iron and thus are especially useful in preventing absorption of the metal that has been indiscriminately added to processed foods. In the Netherlands, a set of 3454 adults, above 55 yrs, were free at baseline of cardiovascular disease [48]. They were observed for 3 yrs for possible development of calcified plaques in the ab-

dominal aorta. With 1–2 cups of green tea per day, plaque development was lowered by 50%; with 4 cups per day, by 67%.

Because of the pervasive addition of readily absorbable forms of non-heme iron to processed foods in the US, tea consumption is strongly indicated. Moreover, the quantity of added iron listed on the labels of processed foods may be erroneous. A US Food & Drug Administration assay of the actual amounts of iron adulteration of dry cereals showed, in some cases, up to 380% higher quantities than that stated on the labels [144].

To ensure excellent respiratory tract health, all sources of inhaled iron should be avoided. A very common source is tobacco smoke [132]. Indeed, was the tobacco plant to be genetically modified so that its leaves would no longer sequester remarkably high amounts of iron, the tobacco product might become quite safe to smoke. Urban air particulates, especially those in subways, are highly contaminated with iron.

Industrial workers exposed to airborne iron and especially coal and iron miners are advised to wear masks. Their on-the-job clothing must be carefully laundered to prevent their family members or laundry workers from being exposed to iron dust. The past indiscriminant use of iron varieties of asbestos has been curtailed. Persons who live near outcroppings of iron-containing deposits of tremolite asbestos are warned to avoid using the mineral as whitewash on the inner or outer walls of their homes [73].

Reduction of body iron by routine blood donation is an effective way for reducing risk of disease. Whole blood contains about 0.5 mg iron/ml. Thus donation of one pint releases 250 mg of iron. This quantity is approximately representative of 50 ng of serum ferritin [28]. Normal menstruation results in excretion of 180–360 mg iron/yr. Non-menstruating women as well as all normal men can maintain low body iron burden by donating blood 2–3 times/yr. Daily ingestion of aspirin, by causing intestinal microbleeding, lowers iron to an extent comparable to that of menstruation.

In a cohort of 181 men followed for five years, blood donations not only lowered iron but increased insulin sensitivity [43]. In a set of 1,277 persons (mean age 67 yrs), those randomized to iron reduction by graded phlebotomy during a six year period had a 36.7% reduced risk of cancer occurrence ($p = 0.023$) and a 66.6% lowered cancer mortality ($p = 0.003$) compared with controls. Reduced cancer risk was observed for most cancer types and occurred over the entire patient age range [28].

For such whole body iron loading conditions as thalassemia, sicklemia and myelodysplasia, phlebotomy cannot be utilized. Thus commercially available iron chelating drugs (deferoxamine, deferiprone, deferasirox) are employed. It would be useful, also, to have iron chelating drugs available for iron normal patients who have cancer, infection or a chronic disorder associated with iron-induced oxidative damage.

Acceptable compounds must have high specificity for iron; low specificity for such other physiologically important metals as zinc, copper and manganese; ability to deplete the iron loaded abnormal site but not iron-normal sites; abstention from redistribution of iron to such iron-sensitive sites as heart or brain; abstention of donation of iron to neoplastic or microbial cells that might be latent in the patient; efficient excretion of the iron chelate in urine or bile; and be available at reasonable cost. The chelator should be employed early in the disease before the damaging effect of oxidative stress has occurred [16].

Among compounds presently being considered for iron withdrawal in neurologic diseases, deferiprone (at one-fourth of dose employed in whole body deironing) has shown possible utility in lowering excessive mitochondrial iron in patients with Friedreich's ataxia [12]. In rodents, VK-28 has provided protection from 6-hydroxy-dopamine pathology [6]. In neuroblastoma cells, degenerative-modifying effects have been obtained with the green tea chelator (-)-epigallocatechin-3-gallate [142]. The comparative properties of low molecular mass iron chelators in clinical use and an evaluation of novel compounds under development have been recently summarized [11].

Two protein iron chelators, transferrin and lactoferrin, now are available for therapy of specific iron loading sites. Transferrin has been extracted from human serum, de-ironed and purified [125]. This product can be used in short-term conditions in which the patient's transferrin saturation is highly elevated. An example is the myelo-ablative conditioning prior to a cell/tissue transplant. The product may be useful, also, in serious cases of bacterial sepsis. Recombinant human lactoferrin is being tested/employed in a considerable diversity of pharmaceutical applications [140]. In most, but not all, the mechanism of action of lactoferrin is considered to be that of iron chelation.

CONCLUSIONS

Excessive/misplaced iron in specific tissues and cells is a prominent risk factor for development of an array

of neurodegenerative and endocrine diseases as well as for cardiomyopathy, arthropathy, neoplasia and infection. Our iron withholding defense system attempts to prevent accumulation of the metal in sensitive sites and to contain it in innocuous packages. The defense system can be compromised by genetic, behavioral and environmental factors. Growing recognition of the ubiquitous iron hazard with increasing use of methods of prevention and therapy can be expected to markedly improve human health.

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Homocysteine, Infections, Polyamines, Oxidative Metabolism, and the Pathogenesis of Dementia and Atherosclerosis

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Abstract. Hyperhomocysteinemia is a risk factor for development of dementia and Alzheimer's disease (AD), and low blood levels of folate and cobalamin are associated with hyperhomocysteinemia and AD. In elderly subjects with cognitive decline, supplementation with folate, cobalamin, and pyridoxal demonstrated reduction of cerebral atrophy in gray matter regions vulnerable to the AD process. Multiple pathogenic microbes are implicated as pathogenic factors in AD and atherosclerosis, and the deposition of amyloid-β (Aβ), phosphorylation of tau protein, neuronal injury, and apoptosis in AD are secondary to microbial infection. Glucose utilization and blood flow are reduced in AD, and these changes are accompanied by down-regulation of glucose transport, Na, K-ATPase, oxidative phosphorylation, and energy consumption. Thioretinaco ozonide, the complex formed from thioretinamide, cobalamin, ozone, and oxygen is proposed to constitute the active site of oxidative phosphorylation, catalyzing synthesis of adenosine triphosphate (ATP) from nicotinamide adenine dinucleotide (NAD^+) and phosphate. Pathogenic microbes cause synthesis of polyamines in host cells by increasing the transfer of aminopropyl groups from adenosyl methionine to putrescine, resulting in depletion of intracellular adenosyl methionine concentrations in host cells. Depletion of adenosyl methionine causes dysregulation of methionine metabolism, hyperhomocysteinemia, reduced biosynthesis of thioretinamide and thioretinaco ozonide, decreased oxidative phosphorylation, decreased production of nitric oxide and peroxynitrite, and impaired host response to infectious microbes, contributing to the pathogenesis of dementia and atherosclerosis.

Keywords: Adenosyl methionine, aging, atherosclerosis, cystathionine synthase, dementia, homocysteine, nitric oxide, oxidative phosphorylation, peroxynitrite, polyamines, thioretinaco ozonide

HOMOCYSTEINE AND DEMENTIA

A prospective study of 1,092 participants in the Framingham Heart Study without dementia demonstrated that those subjects with elevated plasma homocysteine levels have an increased risk of subsequent development of dementia and Alzheimer's disease (AD) after eight years of observation [1]. Previous studies established that low blood levels of folate and cobalamin and elevated homocysteine levels are associated with clinically or histopathologically confirmed dementia of the Alzheimer type [2, 3]. An interventional study to lower homocysteine levels with B-vitamins (folic acid 0.8 mg, vitamin B6 20 mg, vitamin B12 0.5 mg) in elderly subjects with

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mild cognitive impairment demonstrated reduction of cerebral atrophy in gray matter regions specifically vulnerable to the AD process, including the medial temporal lobe, during a 2-year treatment protocol [4]. A recent analysis demonstrated a decreased incidence of dementia over three decades among participants in the Framingham Heart Study [5]. Although the prevalence of vascular disease risk factors (except for diabetes and obesity) associated with dementia has decreased over the period of observation, these trends do not completely explain the observed decrease in dementia [5].

INFECTIONS IN DEMENTIA AND ATHEROSCLEROSIS

Because of the failure of 413 therapeutic trials of AD, based on the amyloid cascade hypothesis, current opinion is that therapeutic intervention based on the microbial etiology of AD is more likely to be successful [6]. A recent review summarizes the evidence for the pathogenesis of AD from chronic infection by *Herpes Simplex Virus, Cytomegalovirus,* other *Herpesviridae, Chlamydophilia pneumoniae,* spirochetes, *Helicobacter pylori,* and various periodontal pathogens [7]. According to this view, the deposition of amyloid-β (Aβ), phosphorylation of tau protein, neuronal injury, and apoptosis are reactive processes caused by chronic microbial infection. Supporting evidence is that Aβ is an anti-microbial peptide deposited in senile plaques and neurofibrillary tangles as a normal function of the innate immune system in AD [8]. Host cell toxicity from anti-microbial peptides induces mitochondrial dysfunction by Aβ peptides by decreasing membrane fluidity [9], opening of the mitochondrial permeability transition pore [10], release of cytochrome C, induction of DNA breaks, and cleavage of poly-ADP ribose polymerase (PARP), the NAD^+-dependent enzyme that is involved in DNA repair [11].

AD accounts for 50 to 56% of cases of dementia in autopsy and clinical series, and AD combined with intracerebral vascular disease accounts for another 13 to 17% of cases of dementia [12]. Aging is the principal risk factor for AD, with the incidence doubling every 5 years after 65 years of age, affecting approximately one third of individuals over the age of 85 [12]. Atherosclerosis is also associated with hyperhomocysteinemia and aging, and blood homocysteine levels increase approximately 1 μmol/L per decade over the age of 60 [13]. Increasing evidence supports the view that microbial infections, including most of the organisms implicated in the etiology of AD, are causal in the pathogenesis of atherosclerosis [14]. The origin of vulnerable atherosclerotic plaques is attributed to obstruction of vasa vasorum by aggregates of microbes and lipoproteins, exacerbated by homocysteinylation of low-density lipoprotein (LDL), production of autoantibodies to LDL, endothelial dysfunction and impaired erythrocyte deformability, resulting in an intimal micro-abscess, the vulnerable plaque [15].

OXIDATIVE METABOLISM AND DEMENTIA

Imaging of subjects with AD using positron emission spectroscopy demonstrates progressive reduction in brain glucose metabolism and blood flow in relation to the severity of dementia [16]. These reductions follow regional synaptic loss or dysfunction, reflecting downregulation of gene expression for glucose transport, Na, K-ATPase, oxidative phosphorylation, and energy consumption in brain [16]. In the process of aging, dysfunctional mitochondria show a decreased capacity to produce ATP by oxidative phosphorylation because of diminished activities of complexes I and IV [17]. In addition to decreased electron transfer and reduced oxygen consumption in mitochondria of aged animals, a decreased membrane potential, increased oxidation products of phospholipids, proteins, and DNA, and increased size and fragility of mitochondria are also observed. In a study of hippocampal neurons in AD, the levels of mitochondrial DNA and cytochrome oxidase-1 were found to be increased, even though the number of mitochondria per neuron is decreased, and evidence of mitosis in pyramidal neurons is interpreted to indicate reactive synthesis of new mitochondria [18].

THIORETINACO OZONIDE AND OXIDATIVE PHOSPHORYLATION

Because of the discovery of failure of malignant cells to oxidize the sulfur atom of homocysteine thiolactone to sulfate [19], a series of synthetic derivatives of homocysteine thiolactone was tested for anti-neoplastic activity in mice with transplanted tumors [20]. The amide synthesized from homocysteine thiolactone and retinoic acid, thioretinamide (TR), was found to have anti-neoplastic, anti-carcinogenic, and anti-atherogenic activity in mice and rats [20, 21].

Two molecules of thioretinamide form a complex with cobalamin to form thioretinaco (TR_2Co), and the disulfonium derivative of thioretinaco, produced by reaction of thioretinaco with ozone, was proposed to catalyze ATP synthesis by the F1F0 complex of mitochondria by stereospecific binding of the phosphate groups of ATP to the disulfonium sulfur atoms of thioretinaco ozonide complexed with oxygen ($TR_2CoO_3O_2ATP$) [20]. The active site of oxidative phosphorylation was proposed to result from binding of nicotinamide adenine dinucleotide (NAD^+) and inorganic phosphate ($H_2PO_4^-$) to form $TR_2CoO_3O_2NAD^+H_2PO_4^-$, which catalyzes ATP synthesis in coordination with reduction of oxygen by electrons from electron transport complexes and production of a trans membrane potential through creation of a proton gradient [22].

ADENOSYL METHIONINE AND HYPERHOMOCYSTEINEMIA

Adenosyl methionine is the sulfonium derivative of methionine formed from the reaction of ATP and methionine [23]. Adenosyl methionine is the allosteric regulator of methionine metabolism which inhibits the activity of methylenetetrahydrofolate reductase [24] and stimulates the activity of cystathionine synthase [25]. Because of these regulatory effects, decreased intracellular concentrations of adenosyl methionine cause increased methylation of homocysteine to methionine and decreased conversion of homocysteine and serine to cystathionine, leading to hyperhomocysteinemia. A proposed scheme for adenosyl methionine synthesis from methionine requires thioretinaco ozonide and ATP [26]. The increasing plasma homocysteine levels and decreasing cellular oxidative phosphorylation observed in human aging are attributed to loss of thioretinaco ozonide from cellular membranes and decreased production of adenosyl methionine [26]. The hyperhomocysteinemia and decreased oxidative phosphorylation observed in AD [1, 16] are attributable to loss of thioretinaco ozonide from mitochondria and dysregulation of methionine metabolism because of decreased biosynthesis of adenosyl methionine [26]. The proposal for the dependence of oxidative phosphorylation upon $TR_2CoO_3O_2$ and NAD^+ suggests that declining NAD^+ and $TR_2CoO_3O_2$ concentrations occur because of loss of these coenzymes from mitochondrial F1F0 complexes during aging [22].

INFECTIONS, POLYAMINE BIOSYNTHESIS, NITRIC OXIDE, AND PEROXYNITRITE

Pathogenic microbes, as observed in brain in AD and in atherosclerotic arterial plaques, synthesize polyamines that are necessary for a broad range of functions, including genetic translation, genetic regulation, resistance to stress, cell proliferation, and differentiation [27]. Polyamines are synthesized in cells infected with viruses [28] and a wide variety of microorganisms [29]. In a recent study, *Chlamydia trachomatis,* the most common agent of sexually transmitted disease, was found to inhibit cellular nitric oxide (NO) synthesis in cultured human mesenchymal stem cells by stimulating polyamine synthesis [30]. Infection by *C. trachomatis* produced downregulation of inducible NO synthase (iNOS) and upregulation of ornithine decarboxylase, which is the rate-limiting enzyme in the polyamine biosynthetic pathway. No studies of polyamine biosynthesis and downregulation of NO by *Chlamydia pneumoniae,* spirochetes, viruses, periodontal pathogens, and other microbes implicated in AD and atherosclerosis have been reported. NO has powerful anti-microbial activity because of formation of peroxynitrite ($OONOO^-$) from superoxide (O_2^-) [31]. Large quantities of NO are produced during infections caused by pathogens, including bacteria, viruses, parasites, and fungi, and peroxynitrite has bactericidal activity which aids in the cytotoxic action of macrophages and neutrophils by inducing nitrative stress and formation of 3-nitro tyrosine [32]. Both reactive oxygen intermediates and reactive nitrogen intermediates are delivered to phagosomes of neutrophils and macrophages to mediate antimicrobial activity against *Chlamydia pneumoniae, Mycoplasma pneumoniae, cytomegalovirus, Staphylococcus aureus*, and other pathogenic microbes implicated in the pathogenesis of AD and atherosclerosis [7, 14, 33].

Increased susceptibility to infectious microbes may occur in aging because of reduced concentrations of thioretinaco ozonide within cellular membranes, resulting in decreased production of nitric oxide, superoxide, and peroxynitrite, potentially explaining the exponential increase in incidence of dementia and atherosclerosis with aging. Recent results demonstrate a role for cystathionine synthase in intracellular NO biosynthesis because of the ability of the heme co-factor of cystathionine synthase to reduce nitrite and generate NO [34]. Studies of the

kinetics of nitrite formation and peroxynitrite formation by ferrous heme implicate cystathionine synthase as a previously unrecognized source of NO and peroxynitrite [35].

The synthesis of the polyamine spermidine is accomplished by transfer of the aminopropyl group of adenosyl methionine to the amino group of putrescine (di-amino butane) [29]. This transfer reaction is catalyzed by S-adenosylmethionine decarboxylase and spermidine synthase (putrescine aminopropyltransferase). Thus microbial infection by a wide variety of microorganisms, including viruses, bacteria, protozoans, and fungi, causes a depletion of adenosyl methionine within host cells because of increased synthesis of polyamines. Decreased adenosyl methionine within infected host cells causes dysregulation of methionine metabolism because of decreased allosteric inhibition of methylenetetrahydrofolate reductase and decreased allosteric activation of cystathionine synthase, resulting in excess production of homocysteine, explaining the hyperhomocysteinemia observed in AD and atherosclerosis [1, 13].

INFECTIONS, OXIDATIVE PHOSPHORYLATION, THIORETINACO OZONIDE, AND AGING

Infectious microbes may deplete host cells of the active site of oxidative phosphorylation, $TR_2CoO_3O_2NAD^+H_2PO_4^-$, because of utilization of this complex for oxidative metabolism by these chronic intracellular pathogens. Depletion of adenosyl methionine from infected host cells by polyamine synthesis [29] will inhibit biosynthesis of thioretinamide because of decreased allosteric activation of cystathionine synthase [25], decreased heme oxygenase activity, resulting in reduced conversion of retinol to retinoic acid by superoxide, and reduced reaction of retinoic acid with homocysteine thiolactone to form thioretinamide [36]. Support for this proposal is the recent observation of reduced concentrations of the cobalamin co-enzymes, methylcobalamin and adenosyl-cobalamin in human brain tissue in aging, autism, and schizophrenia [37]. This study supports the concept of a decreased concentration of thioretinaco ozonide concentration within mitochondrial and cellular membranes as an important factor in the process of aging [26]. In addition, young males with schizophrenia have been observed to have hyperhomocysteinemia [38] and impaired glutathione synthesis, associated with oxidative stress from genetic and functional factors [39].

ENDOTHELIAL DYSFUNCTION, HOMOCYSTEINE, AND ENDOPLASMIC RETICULUM STRESS

Endothelial dysfunction, one of the earliest manifestations of atherogenesis, is promoted by hyperhomocysteinemia [13, 40]. In human endothelial cells homocysteine induces apoptosis through activation of the unfolded protein response, signaled by the endoplasmic reticulum kinase IRE-1 [41]. Induction of endoplasmic reticulum stress by homocysteine causes dysregulation of the pathways for cholesterol and triglyceride biosynthesis, causing fat deposition in liver [42]. The unfolded protein response is clearly established as a factor in the atherogenic effect of hyperhomocysteinemia in production of human and model atherosclerotic plaques, because of a response to endoplasmic reticulum stress, resulting in apoptosis [43]. Herp is an endoplasmic reticulum protein encoded by the *HERPUD-1* (homocysteine-inducible, endoplasmic stress inducible, ubiquitin-like domain member 1) gene, which is induced by homocysteine, facilitates endoplasmic stress, is expressed in neurons and glial cells including astrocytes, and is deposited in the Lewy bodies of neurons and in substantia nigra glial cells in Parkinson's disease [44].

NEUROTOXICITY, MISFOLDED PROTEIN RESPONSE, AND NEURODEGENERATION

Exposure to the neurotoxic amino acid, β-methylamino alanine (BMAA), which is produced by cyanobacteria, is implicated in the etiology of amyotrophic lateral sclerosis (ALS)/Parkinsonism dementia complex in Chamorro patients from Guam [45]. Subsequently BMAA was detected in patients with sporadic AD and ALS from North America, using a validated fluorescent high performance liquid chromatography (HPLC) method with tandem mass spectroscopy for confirmation of BMAA identification [46]. Mis-incorporation of BMAA into human proteins in place of L-serine was found to cause protein misfolding and aggregation in cell cultures [47]. Using a mouse model of the sticky mutation, which is characterized by follicular dystrophy, hair loss, cerebellar Purkinje cell loss, and ataxia, a

missense mutation of the alanyl-tRNA synthetase gene was found to result in low levels of mischarged tRNA molecules, producing misfolded proteins and cell death associated with neurodegeneration [48]. Experimental administration of the neurotoxin BMAA to vervets produces an animal model of AD, characterized by neurofibrillary tangles and amyloid deposits in the brain [49].

ADENOSINE MONOPHOSPHATE KINASE, HOMOCYSTEINE, AND HEPATIC STEATOSIS

Adenosine monophosphate-activated kinase (AMPK) is a metabolic master switch, which controls the metabolic pathways of hepatic ketogenesis, cholesterol biosynthesis, lipogenesis, triglyceride synthesis, adipocyte lipolysis, and fatty acid oxidation by phosphorylation of key enzyme proteins [50]. AMPK is activated by an increased intracellular ratio of AMP to ATP, stimulating oxidative phosphorylation and increased ATP synthesis by mitochondria [51]. In a study of cultured adipocytes, homocysteine was demonstrated to suppress lipolysis by activating the AMPK pathway, resulting in elevation of intracellular triglycerides [52]. In a related study, homocysteine was demonstrated to increase resistin production from adipose tissue in mice with hyperhomocysteinemia and from cultured adipocytes [53]. These studies implicate activation of AMPK in the production of fatty liver in subjects with homocystinuria and in subjects with type 2 diabetes [13].

HOMOCYSTEINE, EXCITOTOXICITY, AND NEURODEGENERATION

Homocysteine is an excitatory neurotransmitter that binds to the N-methyl D-aspartate (NMDA) receptor and leads to oxidative stress, cytoplasmic calcium influx, apoptosis, and endothelial dysfunction [40]. Homocysteine sulfinic acid, an oxidized derivative of homocysteine, is a potent excitatory neurotransmitter, which stimulates glucose uptake through the calcium-dependent AMPK-p38 MAPK-protein kinase C pathway in muscle cells [54]. The $A\beta_{42}$ oligomers that are present in neuritic plaques in AD activate the calmodulin-dependent protein kinase kinase (CAMKK2)-AMPK kinase pathway through phosphorylation of tau protein [55]. Over activation of CAMKK2 or AMPK induces dendritic spine loss in hippocampal neurons of transgenic mice for human amyloid-β protein precursor [55].

ALZHEIMER'S DISEASE, THIORETINACO OZONIDE, AND CANCER

In a study of 1,278 participants in the Framingham Heart Study, survivors of cancer were found to have a 33% decreased risk of developing AD, compared with participants without cancer, and participants with probable AD had a decreased risk of incident cancer, confirming the results of previous studies [56]. These authors considered polymorphisms of p53, the tumor suppressor gene, or Pin-1, a protein which is necessary for cell division and control of protein folding, as possible explanations of this observation. Another possible explanation of this observation is the upregulation of oxidative phosphorylation of impaired neurons which propagates to neighboring cells, promoting cell death in AD [57]. These authors point to the increased glycolysis in cancer cells as a metabolic factor that may explain the observation of an inverse association of AD and cancer. A possible molecular explanation of this inverse association is the depletion of thioretinaco ozonide from malignant cells, leading to aerobic glycolysis, because of proliferation of a clone of cells with loss of the heme oxygenase function of cystathionine synthase and consequent deficient synthesis of thioretinamide, thioretinaco, and thioretinaco ozonide [36]. In AD the depletion of thioretinaco ozonide and consequent impaired oxidative phosphorylation [16] is attributable to decreased biosynthesis of adenosyl methionine because of increased polyamine synthesis by infectious microbes, impairing biosynthesis of thioretinamide by cystathionine synthase and reducing production of thioretinaco ozonide from thioretinamide and cobalamin [29, 36]. In addition, oncogenic viruses may suppress cystathionine synthase function produced by depletion of intracellular adenosyl methionine because of increased polyamine synthesis, allowing a clone of cells with loss of the heme oxygenase function of cystathionine synthase to proliferate. According to this view, carcinogenesis by viruses or carcinogenic chemicals may inhibit oxidative metabolism, reducing the risk of AD because competition for thioretinaco ozonide biosynthesis may suppress the metabolic activity and viability of infectious microbes involved in the pathogenesis of AD.

DETECTION, PREVENTION, AND TREATMENT OF ALZHEIMER'S DISEASE

Early detection of subjects at risk for AD may be accomplished by the finding of mild cognitive impairment through abnormal Mini-Mental State Examination (MMSE) scores, computed tomography or magnetic resonance imaging scans of medial temporal lobe thickness, cerebrospinal fluid $A\beta_{40}$, $A\beta_{42}$, or tau protein, plasma homocysteine, C-reactive protein, and ocular biomarkers [4, 58]. Identification of pathogenic microbes by sero-positivity, positive culture, or other methods will guide the choice of antibiotic, vaccination, or other anti-microbial strategy [7, 59]. Treatment of the metabolic alterations induced by pathogenic microbes in AD and atherosclerosis, including hyperhomocysteinemia, increased polyamine synthesis, and impaired oxidative metabolism from depletion of thioretinaco ozonide, may be addressed by a proposed protocol of thioretinamide, vitamin B complex vitamins, including methyl-cobalamin, methyl-folate, pyridoxal phosphate, and nicotinamide riboside, ascorbate, co-enzyme Q10, adenosyl methionine, menoquinone, amygdalin, vitamin D3, pancreatic enzymes, cod liver oil, and dietary improvement to eliminate processed foods and to prevent subclinical protein energy malnutrition [13, 60]. In addition, meticulous oral hygiene, consumption of dietary monolaurin and other nutrients with anti-microbial activity, consumption of adequate dietary protein, and avoidance of neurotoxins from foods or environmental contaminants may also retard the progression of mild cognitive impairment to dementia [61, 62]. The efficacy of this proposed protocol requires validation by a properly designed clinical trial [63].

CONCLUSION

The metabolic abnormalities caused by infectious microbes in dementia and atherosclerosis affect homocysteine metabolism, oxidative phosphorylation, and biosynthesis of polyamines, leading to neurodegeneration and atherosclerotic arterial plaques. These abnormalities consist of decreased concentrations of thioretinaco ozonide, adenosyl methionine, and reduced allosteric activation of cystathionine synthase in host cells. These metabolic changes impair the host response to infectious microbes because of impaired production of nitric oxide and peroxynitrite in macrophages. A proposed strategy for prevention and treatment of dementia and atherosclerosis consists of early detection of cognitive decline, dietary improvement to eliminate highly processed foods, adequate dietary protein to prevent subclinical protein energy malnutrition, dietary consumption of anti-microbial nutrients, meticulous oral hygiene, and a homocysteine-lowering protocol consisting of thioretinamide, B vitamins, coenzyme Q10, ascorbate, adenosyl methionine, menoquinone, amygdalin, vitamin D3, cod liver oil, and pancreatic enzymes.

DISCLOSURE STATEMENT

The author's disclosure is available online (http://j-alz.com/manuscript-disclosures/16-0549r1).

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Apolipoprotein E Related Co-Morbidities and Alzheimer's Disease

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Abstract. The primary goal of advancement in clinical services is to provide a health care system that enhances an individual's quality of life. Incidence of diabetes mellitus, cardiovascular disease, and associated dementia coupled with the advancing age of the population, have led to an increase in the worldwide challenge to the healthcare system. In order to overcome these challenges, prior knowledge of common, reliable risk factors and their effectors is essential. Oral health constitutes one such relatively unexplored but indispensable risk factor for aforementioned co-morbidities, in the form of poor oral hygiene and tooth loss during aging. Behavioral traits such as low education, smoking, poor diet, neglect of oral health, lack of exercise, and hypertension are few of the risk factors that are shared commonly among these conditions. In addition, common genetic susceptibility traits such as the apolipoprotein E gene, together with an individual's lifestyle can also influence the development of co-morbidities such as periodontitis, atherosclerosis/stroke, diabetes, and Alzheimer's disease. This review specifically addresses the susceptibility of apolipoprotein E gene allele 4 as the plausible commonality for the etiology of co-morbidities that eventually result from periodontal diseases and ultimately progress to dementia.

Keywords: Alzheimer's disease, apolipoprotein, atherosclerosis, co-morbidities, dyslipidemia, periodontitis

THE CONCEPT OF SUCCESSFUL AGING

Successful aging describes optimization of life expectancy while minimizing physical and mental deterioration and disability. Such a state would be characterized by good health and high levels of independent performance and cognitive functioning [1]. Absence of disease would include chronic diseases such as periodontitis (PD), cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), and Alzheimer's disease (AD), all of which have an impact on an individual's longevity and quality of life. Even if PD was manifested by an individual, but the disease process was controlled by regular dental prophylaxis, 'successful aging' would still be measured by having retained a greater number of an individual's own teeth [2, 3]. Interestingly, retention of teeth has been positively associated with higher cognitive functioning in the elderly [4]. Further support comes from longevity in the very elderly subjects, referred

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to as the centenarians, who appear to bypass dementia [5–7] by circumventing other conditions such as diabetes and CVD [8], supporting the potential association of multiple-co-morbidities in the development of dementia.

According to the focal infection theory [9, 10], the polymicrobial dysbiosis of PD [11] and the subsequent host's immune responses [12] are the pivotal factors that bind the eclectic mix of conditions ranging from the oral condition and T2DM, to inflammatory pathologies including vascular disease(s) and AD. The apolipoprotein gene allele 4 (APOE ε4) is a susceptibility gene, the inheritance of which not only predisposes individuals to infections [13] that initiate inflammation, but also cause disturbances in their lipid metabolism resulting in dyslipidemia [14]. However, our own vision of how a risk factor such as an infection may lead to co-morbid states is illustrated in Figure 1.

APOE ε4 has recently been implicated in the aggressive form of periodontitis [15] and in a more aggressive onset of AD [16–18]. Specific microbes such as *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) is associated with localized aggressive periodontitis in children and teenagers [19, 20]. The age of onset with plausible genetic factors and the host's immune response predisposing an individual to early (aggressive) or late (chronic) onset PD [21, 22] suggests that both forms of periodontitis may eventually become recognized as one disease entity [23].

Thus, APOE ε4 with an environmental risk factor such as an infection, and/or a fatty diet, combined with smoking and sedentary lifestyle, will likely enhance its biological function in favor of disease outcome. Given that the APOE ε4 is linked to several diseases such as PD, T2DM, CVD, and AD [15–17, 24–28], all of which demonstrate an element of inflammation and dyslipidemia in their pathogenesis [29–33], further supports the role for infections [25] as a dominant environmental response modifier of disease states. With the growing interest in co-morbid states as well as with the possible association between PD via infections and lifestyle behaviors, it is of interest to explore APOE and its allelic variants further.

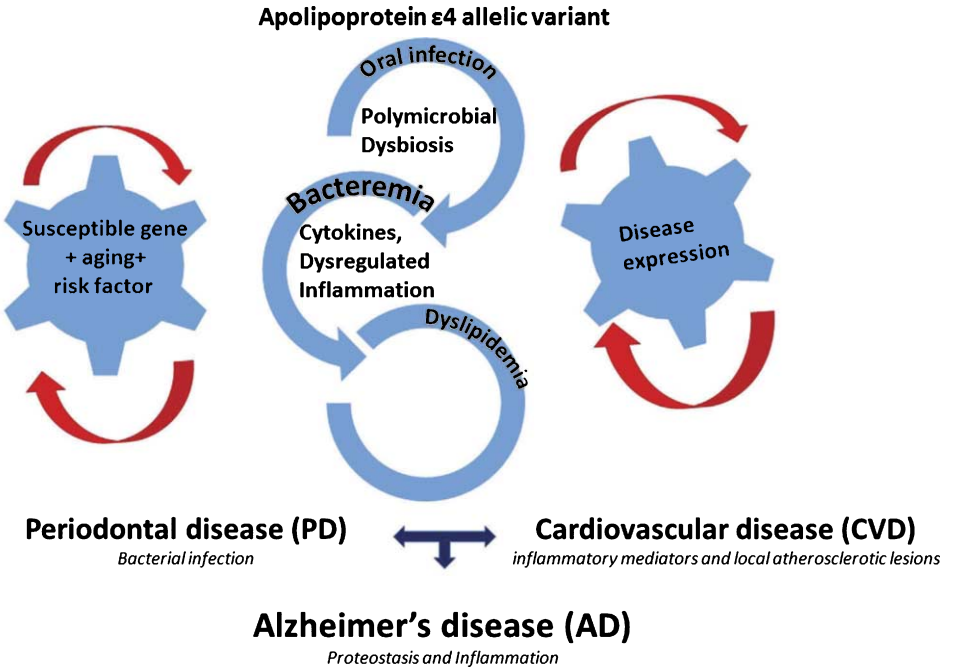

Fig. 1. Schematic illustration of the intricate cascade of interaction between APOE ε4 and environmental risk factor such as an oral infection from PD. Following poor oral hygiene the gingivae can bleed and allow access of periodontal bacteria to the systemic circulation where immune cells survey entry of noxious agents. Upon recognizing pathogenic bacteria, these immune cells release inflammatory mediators (cytokines) to combat infection but as the pathobionts have strategies of their own to evade the immune surveillance they remain viable. At the acute phase of infection, disturbances in the lipid metabolism take place in the form of dyslipidemia. If the lipid imbalance is sustained, during aging, the dyslipidemia can augment disease pathogenesis including atherosclerosis, CVD, and AD.

APOLIPOPROTEIN E

ApoE is a 34 kDa plasma lipoprotein and its gene (APOE) is located on the long arm of chromosome 19 (q) at position 13.2 [16, 17]. The protein structure of ApoE shows two structural domains [34] that are held together with a hinge region [35] in the three human allelic (ε2, ε3, and ε4) variants [36] (Fig. 2). The proteins of these allelic variants differ by virtue of two amino acid substitutions at the 61 and 112 amino acid positions. For example, ε2 has cysteines (Cys-61 and Cys-112), ε3 has Arg-61 and Cys-112, and ε4 has arginine (Arg-61 and Arg-112) at both positions [34]. The amino acid Cys-112 in both ε2 and ε3 preferentially bind high-density lipoproteins (HDL) whereas Arg-112 in ε4 preferentially binds the very low-density lipoprotein (VLDL) lipoproteins [34]. The amino acid change in ε4 considerably alters its structure with impact on its domain interaction (Fig. 2) and subsequently function in favor of diseases [34, 37, 38] associated with an element of dyslipidemia in their pathogenesis.

Briefly, dietary fat is converted into fatty acids largely by the various lipase enzymes aiding their digestion [39]. The simplified fatty acids are eventually absorbed by the intestinal mucosa and released into the blood stream in the form of chylomicrons [39]. These plasma lipoproteins based on their relative content of cholesterol and triglycerides are classified into four major classes such as chylomicrons, VLDL, low-density lipoproteins (LDL), and HDL. ApoE within the blood plasma acts as a form of transport for phospholipids and the nonpolar lipids such as cholesterol and triglycerides to remote body locations. Any surplus lipids are returned to the liver where they undergo several biochemical reactions for either storage as adipose tissue or conversion into vitamin D and appropriate hormones [40]. Any surplus LDL over and above its storage capacity in the blood stream is deemed harmful as it initiates atherosclerosis [41].

ApoE is abundantly synthesized by the hepatocytes in the liver [36] and in the brain, predominantly by astrocytes for local needs [42, 43]. While ε2 appears to be rarely inherited, it is associated with the genetic disorder known as type III hyperlipoproteinemia. ApoE ε3 is the most common isoform found in humans [44] and is considered to be the normal form [38]. ApoE ε4 appears to be associated with the metabolic disorder T2DM [27] and various inflammatory pathologies including aggressive periodontitis [15], CVD [24, 26], and AD [16, 17, 25, 28]. ApoE ε4 may therefore, be interfering with the phenomenon described as 'successful aging' processes

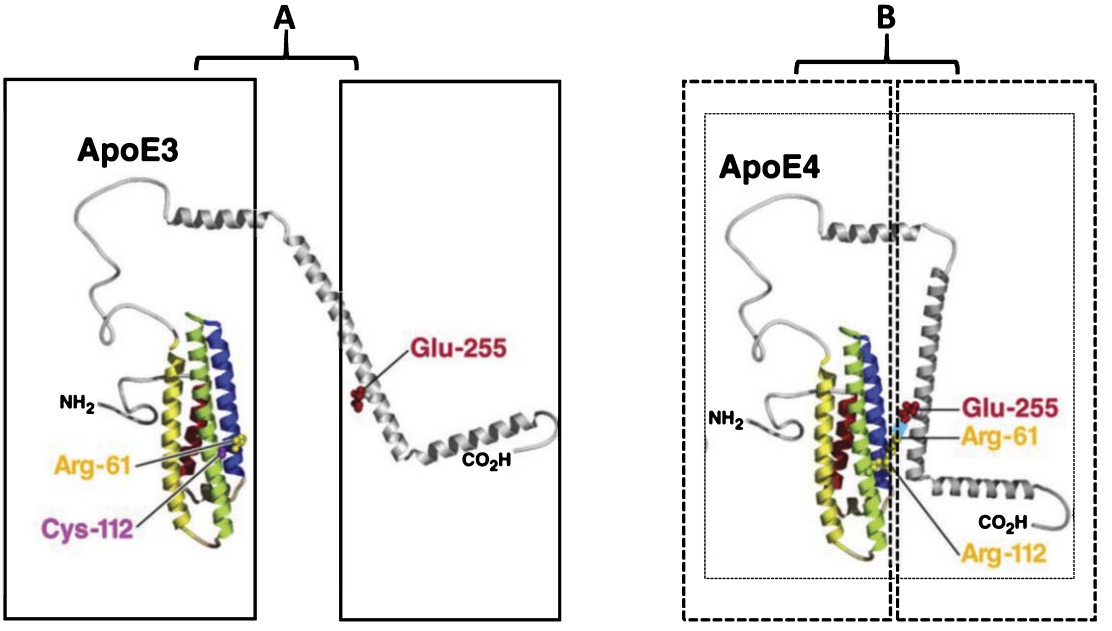

Fig. 2. Models of ApoE3 and ApoE4 adapted from Mahley and Huang [149]. The rectangular boxes show the differences in the molecule in respect to amino acid changes in the two allelic variants. The space between the two boxes (solid lines) in A is greater than in B (boxes with broken lines) where domain interaction appears restricted.

[1] via dyslipidemia and behavioral traits. The main focus of this review is to envisage the plausible common risk from ApoE ε4 in these aforementioned co-morbid states, from periodontitis to AD in relation to oral pathobionts.

PERIODONTAL DISEASE

PD is a polymicrobial dysbiotic inflammatory disease of the tooth supporting structures, characterized by the destruction of the gingival connective tissue attachment to the root surface and adjacent alveolar bone. Over 700 different bacterial species have been identified in the oral cavity of humans, 400 of these are from the subgingival sulcus [45]. Of the 400 phylotypes of subgingival microbiota, PD involves interaction of specific bacteria; *A. actinomycetemcomitans*, *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*) and *Tannerella forsythyia* (*T. forsythia*) [11, 46] and are considered major contributors of human periodontal disease(s) [11, 47].

Disease progression depends on the host's inflammatory and immune responses to the pathogens [12]. As a consequence of host-pathogen interaction, low-grade inflammatory mediators are continuously being released [48] and these locally breach the periodontal pocket integrity exposing vascular channels to a flow of inflammatory mediator rich sustenance, favorable for the exponential growth of subgingival microflora. Destruction of host gingival tissues is the consequence of this exposure [49, 50]. Incidence of transient bacteremia following chewing, tooth brushing, and scaling in individuals with periodontal inflammation [51], enabling oral bacteria and bacterial components hematogenous to several systemic organs. Poor oral hygiene, and genetic susceptibility with ApoE and low-density lipoprotein receptor-related protein 5 (LRP5) polymorphisms and in the neuropeptide Y (NPY) gene in aggressive periodontitis in the susceptible male host (whereas it is downregulated in female subjects) have been identified suggesting a sex-specific effect of genetic variation of NPY on PD [52]. Genetic polymorphisms would appear to be a risk factor in developing PD, which subsequently associates with remote organ metabolic states such as diabetes [53], and inflammatory pathologies such as vascular disease(s) [54, 55], and AD [56–61], and others that are out of the scope of this review.

Nutrition plays an important role in the development of PD. Poor nutrition, specifically foods high in dietary cholesterol or fatty acids, inhibit the immune system [62]. However, it remains unclear whether it is abnormal lipid metabolism or dyslipidemia that leads to PD or PD leads to impaired lipid metabolism [63]. Dyslipidemia frequently results from infections that initiate release of inflammatory mediators in the form of cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), and interferon-gamma (IFN-γ) that increase serum triglyceride levels and suppress fatty acid oxidation [14, 64]. Our own vision of how cytokines from periodontal infection in the susceptible host may lead to disturbances in lipid metabolism is illustrated in Figure 3.

The case-control study of Gao et al. [15] described four important findings in relation to LRP and dyslipidemia in the Chinese PD patients. These are: Individuals with generalized aggressive periodontitis showed significantly lower total cholesterol and lower HDL than controls; and individuals with LRP5 SNPs (rs682429-AA or rs312016-GG) showed higher total cholesterol, higher HDL, and decreased odds for aggressive periodontitis; and individuals with combined polymorphisms (LRP5-rs682429-AA and APOE-rs429358-CC/CT) had high serum LDL and total cholesterol and decreased odds for aggressive periodontitis; and individuals with LRP5 haplotype (rs682429-rs312016:A-G) had decreased odds for aggressive periodontitis.

LRP5 is a co-receptor of the Wnt/β-catenin signaling cascade [65] that in health affords protection to the individual from vascular diseases as demonstrated in ApoE and LRP5 double gene knockout ($ApoE^{-/-}$ $LRP5^{-/-}$) mice [66]. Since LRP5 polymorphisms are also being discovered in aggressive PD, this implies that these polymorphisms are contributing to loss of gene function, and thereby predispose individuals to periodontitis [15]. A plausible mechanism is via association of PD with lower levels of HDL cholesterol and higher levels of both LDL cholesterol and plasma triglycerides [15, 67–69].

Hyperlipidemia, specifically higher total cholesterol and LDL levels, have been reported with periodontitis experimentally, but epidemiological findings have so far contradicted this finding [69]. As periodontal treatment is known to have a beneficial role on lipid metabolism and supports their intricate association, a plausible confounding factor in the Machado et al. [69] study may reflect a mixed

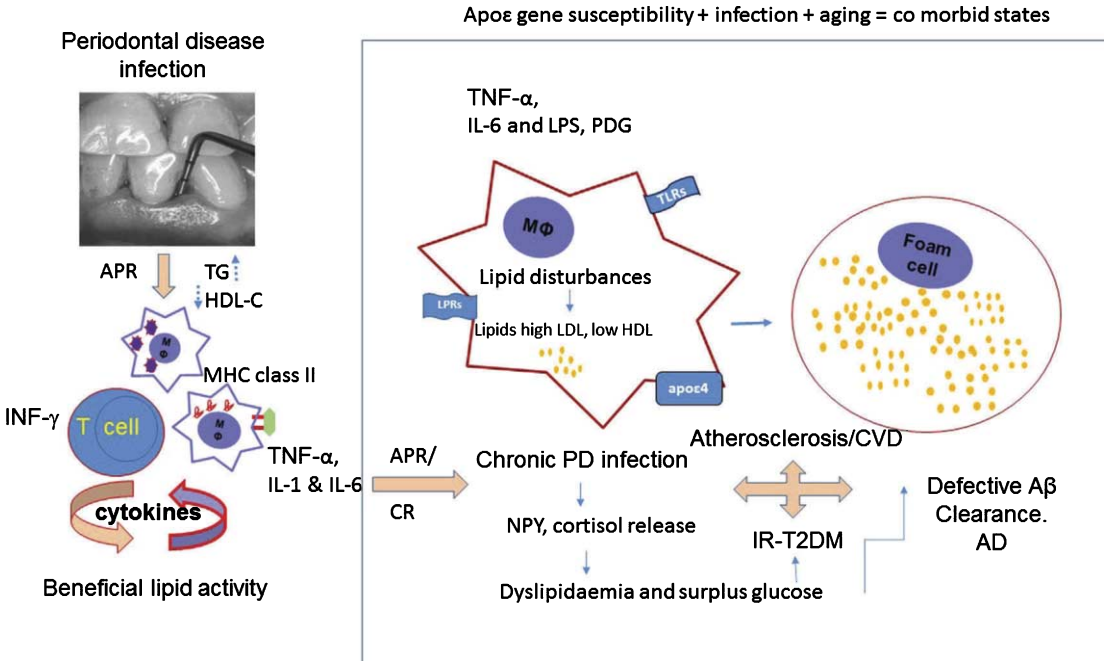

Fig. 3. Pictorial illustration of interaction between APOE ε4 with its infectious risk factor resulting from PD. Following poor oral hygiene, there is local inflammation in the gingivae and in the systemic circulation. The blood borne immune cells at both tissue sites and in the systemic channels release inflammatory mediators (cytokines = APC or acute phase response) to prevent spread of infection. At the same time disturbances in the lipid metabolism take place thereby the balance of LDL and cholesterol becomes "tilted" leading to higher HDL (dyslipidemia). If the lipid imbalance is sustained for longer time, that can augment disease pathogenesis such as atherosclerosis and other co-morbidities. TG, triglycerides; CR, chronic response; MΦ, macrophage; LPS, lipopolysaccharide; PDG, peptidoglycan; TLRs, toll like receptors; LPRs, low density lipoprotein receptors; NPY, neuropeptide Y; IR, insulin resistant; Aβ, amyloid-β. Cytokines (TNF-α, IFN-γ, IL-1, and IL-6), lipids (HDL-C, LDL, HDL), and diseases (PD, CVD, T2DM, AD) are abbreviated as in main body text.

population of individuals taking part; who regularly receive dental treatment alongside those who rarely visit the dentist.

Periodontal disease in ApoE$^{-/-}$ mice

There has been heightened interest in the use of ApoE$^{-/-}$ mice as a model to investigate the association between PD and atherosclerosis, and hence it is vital to obtain an understanding of the role of periodontitis and its inflammatory mediators. PD is classically initiated by the colonization/infection of the periodontal pathogens via the oral route; to this end, various researchers have investigated the effects of oral infection of ApoE$^{-/-}$ mice with periodontal pathobionts (*P. gingivalis, T. denticola, T. forsythia, Fusobacterium nucleatum*) [70–73], both as a polymicrobial infection and as mono-infections [71–74]. These studies have demonstrated bacterial colonization and progression of PD in the ApoE$^{-/-}$ mouse model (bacterial invasion, gingival inflammation, apical migration of junctional epithelium, alveolar bone resorption, and intra-bony defects). By comparing control to infected mice, a significantly elevated IgG response to *P. gingivalis* and *T. denticola* and *T forsythia* mono-infections as well as in the polymicrobial infections was recorded [70–72]. The humoral response generated in all of the infected groups, provides further evidence of a stable response to PD pathogens as well as manifestation of chronic inflammation [70–72]. This primary environmental risk factor (infection) has the potential for pathogenic interplay in the hetero/homozygous ApoE ε4 genotype via initiation of an intrinsic cascade of risk factors (infection>inflammation) for dyslipidemia. Another common feature of all the mono- and poly-infected experiments in ApoE$^{-/-}$ mice [71, 75] was the abundant expression of NPY gene in vascular tissues [75]. This suggests an intricate relationship of NPY gene and chronic infections with possible manifestation for the development of insulin resistance as discussed below.

TYPE 2 DIABETES MELLITUS

Type 2 diabetes mellitus (T2DM) is a metabolic disorder diagnosed in adulthood [76]. It is associated with obesity and is caused by an inefficiency or resistance of the cells to utilize insulin, resulting in a slow but an excess accumulation of sugar in the blood [76]. Insulin resistance reduces glucose tolerance, especially in adipocytes and muscle cells, where the uptake of glucose is insulin dependent. This results in accumulation of glucose in the circulation and a hyperglycemic state [76], and a homeostatic and systemic imbalance, which is detrimental to health [76]. Increasing evidence supports a bidirectional relationship between T2DM and PD [77]. The hyperglycemia associated with diabetes results in an increased deposition of advanced glycation end products (AGEs), which bind to neutrophils inhibiting their normal activity [78]. In addition, AGE products activate its receptor (RAGE), which further alters normal macrophage function [78]. These factors subsequently result in an uncontrolled production of proinflammatory cytokines which eventually cause dyslipidemia as well as increased vascular permeability, collagen fiber break down, and destruction of connective tissue and bone [78, 79]. This may be another mechanism that increases the risk of the diabetic patient to the development of PD.

The ε4 variant of the APOE gene also appears to be associated with T2DM as demonstrated by Alharbi et al. [27] in a Saudi population. The differences between T2DM patients and controls for the homozygous ε4 [E4/E4: OR, 4.39 (95% CI: 2.16–8.92); $p = 0.0001$] were shown to be significant. Since patients with this hetero/homozygous APOE ε4 genotype are predisposed to infections [13] generally and to oral pathobionts due to the bidirectional relationship of PD with diabetes [77], a chronic inflammatory (cytokines) state in the insulin resistant patient is likely. In addition, NPY is upregulated following PD infection as demonstrated by Chukkapalli et al. [75] in ApoE$^{-/-}$ mice. This is significant as NPY during health modulates a multitude of hypothalamus pituitary adrenal (HPA) axis functions via cortisol release including apatite regulation [80], learning and memory [81, 82], mood [83], and neuroprotection [84]. The HPA axis helps to maintain a sustained stress response if the brain continues to sense that a threat, such as an infection, is present in the body. In response, the hypothalamus secretes corticotropin-releasing hormone, which stimulates the pituitary gland to release adrenocorticotropic hormone, and signals to the adrenal glands to increase the levels of circulating cortisol in the blood. Cortisol helps the body to access the resources needed for a sustained response to threat such as maintaining high levels of blood glucose. The individuals having inherited the heterozygous or homozygous APOE ε4 allelic variant metabolise glucose at a lower rate than those with APOE ε2 and APOE ε3 [85]; and the inflammatory mediators contribute to insulin resistance and disturbance in lipid and glucose metabolism [79]. As a result, the function of various tissues and cells, including adipocytes, hepatocytes, muscle and endothelial cells are affected and impaired, which then leads to other chronic metabolic disease states including obesity, CVD, stroke, and AD.

Insulin resistant T2DM in ApoE$^{-/-}$ mice

ApoE$^{-/-}$ mice have not been used as a model for inducing insulin resistance T2DM. Nevertheless, there is a suggestion of an emerging role of NPY gene that may be of relevance to this metabolic syndrome via its effect on the HPA activity as demonstrated by ApoE$^{-/-}$ mice following an oral infection [75].

CARDIOVASCULAR DISEASE

Cardiovascular disease(s) is characterized by the process of atherosclerosis within blood vessels [86]. It can lead to myocardial infarction, stroke, or peripheral arterial disease according to where it manifests within the coronary artery tree, cerebral arteries, and/or peripheral arteries [86]. Inflammation and inflammatory processes leading to dyslipidemia in the vessel wall are major contributors of atherosclerosis [87]. Cardiovascular risk factors show overlapping features with other inflammatory pathologies such as PD and vascular dementias encompassing both lifestyle and genetic factors. These include hypertension, diabetes, dyslipidemia, smoking, and others [88]. The LDL receptor protein mutations and the APOE gene are known genetic susceptibility genes in coronary heart disease [89, 90]. Patients with poorly controlled PD show high levels of circulating C-reactive protein (CRP) and fibrinogen levels in their serum [91, 92]. Since CRP is a predictor of heart disease, its rise during episodes of poor dental hygiene is currently the strongest link between PD and atherosclerotic vascular disease. Translocation of oral pathogens into the main arterial vessels is reported by many investigators using sensitive polymerase

chain reaction (PCR) and sequencing alongside the fluorescence *in-situ* hybridization (FISH) technique [93–97]. These include *P. gingivalis* and *T. denticola* located within the walls of human coronary artery and atheromatous plaque lesions [93–97]. However these studies remain qualitative as it would take a considerable sample size to determine if there is any statistical significance in these findings.

Experimental periodontitis in ApoE$^{-/-}$ mice initiate systemic disease pathology

Previous studies have defined the underlying concepts behind the potential causal-association between microbial agents and atherosclerosis, based on the exacerbation of a chronic inflammatory response largely mediated by bacteria. Recent studies exploring the susceptibility of ApoE$^{-/-}$ mice to atheroma formation with mono-infections or as polymicrobial infections demonstrated that oral, metabolically active, pathogens are able to initiate and sustain atherosclerotic lesions in the aorta [70–72, 74, 75, 98]. Furthermore, Hayashi et al. [98] reported that *P. gingivalis* exposure results in an increase of atherosclerotic plaque accumulation in the innominate artery, which is associated with the accumulation of lipids and macrophages closely mimicking the pathology of the human atherosclerosis.

ALZHEIMER'S DISEASE

AD is a neurodegenerative condition characterized by an irreversible memory deficit. The main neuropathological hallmark proteins are amyloid-β (Aβ) and the hyperphosphorylated microtubule associated intraneuronal neurofibrillary tangles (NFTs), both of which are critical to AD postmortem diagnosis [99]. There are two main forms of AD, the more rare inherited form and the more prevalent, late-onset form. The familial form is characterized by missense mutations in three genes; the amyloid precursor protein (APP), located on chromosome 21, and the presenilin 1 (PSEN 1) and 2 (PSEN 2) genes located on chromosomes 14 and 1, respectively, and are all related to enhanced Aβ deposition. Mutations in the tau gene have been identified in familial forms of frontotemporal dementias linked to chromosome 17 [100] but not in AD that are directly attributed with the NFT lesion. For both forms of AD, there are two major but common risk factors namely advancing age and the APOE ε4 susceptibility gene [16, 17].

Investigating the pathological interactions of mutated genes revealed that the insoluble, fibrillary Aβ plaques are a breakdown product of the APP gene proteases known as α-, β-, and γ-secretases [101]. These proteases are the translational products of PSEN 1 and 2 genes, and the cleavage sites of their substrate (AβPP) are well documented by numerous reviews [101–103]. In essence, the α secretase cleavage of AβPP confers little pathogenicity; whereas; depending on the cleavage site of AβPP by the β- and γ-secretase enzyme(s), two major species of fibrillary $A\beta_{(40/42)}$ are deposited in AD brains. Of these, $A\beta_{42}$ is regarded as the pathogenic form due to its association with neuritic plaques, which are composed of degenerating nerve tissues with a tight core made up of $A\beta_{42}$ fibrils [104]. The toxicity of $A\beta_{42}$ fibrils can be explained by their antimicrobial properties [105]. In the brain, Aβ fibrils play a role as immune modulators of the innate immune system potentiating activation of the complement cascade [106]. Since neurons are vulnerable to complement mediated lysis [107], the neurites on the periphery of $A\beta_{42}$ deposits represent debris of dead neurons whilst glia [108] continue, albeit in vain, to synthesize inflammatory components for their clearance.

Despite the generally accepted toxicity of the fibrillary $A\beta_{42}$, Braak and Braak [109] questioned its correlation with progressive cognitive decline in AD cases. To this end, researchers examining how amyloid fibrils form, led to the simultaneous publication of papers from two laboratories reporting the discovery of 'protofibrils' [110, 111]. Continued work by others has revealed progressively smaller neurotoxic assemblies known as 'oligomers', which appear more toxic than fibrils alone [110–116]. Among these is the soluble form of Aβ*56 that has been shown to be negatively associated with cognitive decline in an AβPP transgenic mouse model [117] and when injected into the rat brain [118]. Consequently, the original amyloid hypothesis of Hardy and Selkoe [113] has been modified to the 'Aβ oligomer hypothesis' as originally termed by Ono et al. [115]. Since both insoluble Aβ plaques and NFT lesions are essential for the definitive diagnosis of AD, the weakness of the amyloid hypothesis remains in demonstrating the association of Aβ with many other pathogenic domains of this specific neurodegenerative condition.

ApoE ε4 has so far emerged as the most significant risk factor for both the familial and late-onset forms of AD associating with almost every pathogenic domain as well as an aggressive disease form with an earlier

age of onset [16, 17, 119]. ApoE ε4 binds Aβ at the 244–272 residue site (C-terminal residues on ApoE ε4) [17, 44]. It has recently been demonstrated that the N-terminal residues of ApoE ε4 bind to NFTs [103] highlighting the important role of this protein in both AD and in the two main pathological lesions (Aβ and NFTs), thereby gaining support for its association in AD proteostasis. Furthermore, the full length ApoE ε4 is prone to proteolytic cleavage at the C-terminus (methionine 272 or serine 268) that produces a 29 kDa fragment and again at the N-terminal resulting in fragments of 14–20 kDa [38]. The partial proteolytic cleavage of ApoE ε4 at the hinge region that holds the two domains together [35] by the yet unidentified proteases has two direct implications in the brain; first the generation of two toxic fragments and second, reduced levels of the whole (ApoE) protein [38]. The reduced levels of ApoE ε4 is unable to maintain adequate lipid homeostasis in the aging brain due to its rapid clearance [120, 121] and its decreased binding to Aβ contributes to amyloid accumulation in AD [44, 122–124] possibly resulting as a form of dyslipidemia.

Infections and inflammation induce dyslipidemia [14], and AD pathogeneses is not complete without documenting chronic peripheral infections [61]. These include *Chlamydophila pneumoniae*, *T. denticola*, and *P. gingivalis*, which are also found in atheroma plaque tissues [93–97] and in AD brains [56, 60, 125], herpes simplex virus type I [126], and several species of spirochetes of which the well cited ones are *T. denticola* [56] and *Borrelia burgdorferi* [127]. Although the exact etiological agent(s) responsible in late-onset AD remain elusive, spirochetes appear as highly plausible candidates as exemplified by the condition long-standing, stationary, or atrophic form of general paresis, which is caused by *Treponema pallidum* infection. The atrophic form of general paresis has recently become accepted as an example of a chronic bacterial infection leading to dementia, reproducing the neuropathological hallmarks of AD [128]. More recent reports relating infections to a causative role in the onset of dementia are supported by Kamer et al. [129] suggesting that mild periodontitis is associated with higher brain amyloid load in normal elderly subjects in the hippocampus.

Dementia can result from infections with AD hallmark proteostatsis [128] and from infection and inflammation alone as exampled by HIV-dementia [130]. The introduction of successful antiretroviral medication has led to people with HIV infection living longer. This is introducing an aging group suffering sustained HIV-associated immune activation and chronic inflammation which is thought to be, at least in part, responsible for the increased comorbid chronic disease that this group experiences. HIV positive subjects show increased prevalence of CVD, hypertension, renal disease, diabetes, and osteoporosis compared to controls [131] and develop HIV-dementia [130].

Experimental periodontitis in ApoE$^{-/-}$ mice initiate inflammatory pathology in the brain

The downstream effects of *P. gingivalis* mono-infection in ApoE$^{-/-}$ mice was recently reported by Poole et al. [132] in which they reported the translocation of this PD pathogen from the oral cavity into the brain tissue likely via the hematogenous route; although other pathways for its translocation are also possible [133]. Examination of the brain tissue highlighted the brains own inflammatory cells (microglia and astrocytes) were activated and neurons were being attacked by excessive complement activation supporting ongoing intracerebral inflammation in the absence of AD hallmark proteins [132]. For the relevance of finding *P. gingivalis* in the ApoE$^{-/-}$ mice brains, the reader is directed to another review article published elsewhere [134].

APOE ε4 AS THE PLAUSIBLE COMMONALITY FOR THE ETIOLOGY OF CO-MORBIDITIES

All of the above mentioned conditions share at least one common genetic susceptibility the ε4 allelic variant, and the common lifestyle and behavioral traits [15, 16, 24, 27]. They all show an association with peripheral infections directly or indirectly [11, 31, 56, 60, 135], inflammation [12, 31, 135, 136], and dyslipidemia [29, 30, 32, 33].

In view of the apparent relationship between successful aging and APOE alleles, it was of interest to explore the association between aging and retention of natural teeth [4]. When APOE allele frequencies were analyzed and compared between groups of edentulous and dentate human subjects, the edentulous group showed a significantly higher frequency of the APOE ε4 allele [4]; but the limitations with this study were that it is unknown whether possessing the APOE ε4 allele made an individual more susceptible to periodontitis specifically, or to disease in

general and tooth loss was a consequence of an overall deterioration in health.

Borilova Linhartova et al. [32] investigated the association between PD and the APOE ε4 allele in a case-control study using genomic DNA in which they reported that APOE gene variability was not significantly different between the two groups examined (chronic PD sufferers and those without PD); although those with chronic PD demonstrated increased total cholesterol and LDLs compared to controls [32]. In addition, no significant differences were found between groups for triglyceride and HDL levels [32]. Although environmental influences such as smoking, age and gender, socioeconomic factors, obesity, diabetes, and family history are known to associate with PD [137–139], the genetic links on the whole, are only now being documented. It is generally recognized that the aggressive forms of PD have a stronger genetic association [15, 22] than the chronic form. However, the research by Gao et al. [15] demonstrated an association between the APOE gene and LRP5 polymorphism in the aggressive form of PD, which along with this genetic risk factor strengthens the periodontal association with the emerging cardiovascular and AD pathologies.

Literature supports the presence of groups of individuals who are destined to suffer, as in, familial forms of disease. These can be excluded from those who inherit susceptibility genes. In addition to APOE ε4 commonality for the etiology of co-morbidities, there are individuals with other common susceptibility traits for PD. These account for approximately 50% genetic variance with polymorphisms in inflammatory mediator gene regions such as IL-1, IgG Fc receptor, and TNF-α. Polymorphisms in IL-1α, IL-1β, IL-6, and TNF-α, complement component 1(q subcomponent, A chain) genotypes are reported in periodontitis [140–143]. Additionally, IL-1α, IL-1β, IL-6, TNF-α, α2-macroglobulin (also known as LDL receptor related protein or LRP), and alpha1antichymotrypsin, complement receptor 1 (CR1) and clusterin are not only all upregulated but also show polymorphic associations in AD cases [144–147] suggesting common inflammatory gene susceptibility profiles in the expression of PD to AD likely contributing to dyslipidemia. As the susceptibility gene clearly requires an environmental risk factor for the expression of disease, avoidance of risk would be one therapeutic solution. For example, it is documented that not everyone with the hetero/homogeneous inheritance of APOE ε4 will result in manifesting diabetes, vascular diseases, and AD [8, 148], and if this risk factor is an oral infection [56, 60, 61], as supported by the ApoE$^{-/-}$ mice induced with PD studies [70–73, 75, 132], then there is a therapeutic window for the related co-morbid states to modify the course of disease by adoption of healthy lifestyles and promotion of awareness about important early warning signs of serious health conditions by regular dental visits.

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Section 8
Gene signature and environmental factors in Alzheimer's disease

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Gene Signature in Alzheimer's Disease and Environmental Factors: The Virus Chronicle

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Abstract. Genome wide association investigations from large cohorts of patients with Alzheimer's disease (AD) and non demented controls (CTR) showed that a limited set of genes were associated ($p > 10^{-5}$) with the disease. A very recent study from our group showed that an additional limited group of SNP in selected genes were associated with AD. In this report we argue that the association of these genes with AD is suggestive of a pivotal role of environmental factors in the pathogenesis of the disease and one of these factors is virus infection. In other words, the genetic signature revealed by genome wide association (GWA) studies discloses a network of genes that might influence the ability of the central nervous system to cope with and fight against the invasion by virus of the herpes family. In fact, Nectin-2 (NC-2); apolipoprotein E (APOE); glycoprotein carcinoembryonic antigen related cell adhesion molecule-16 (CEACAM-16); B-cell lymphoma-3 (Bcl-3); translocase of outer mitochondrial membrane 40 homolog (T0MM-40); complement receptor-1 (CR-l); APOJ or clusterin and C-type lectin domain A family-16 member (CLEC-16A); Phosphatidyl inositol- binding clathrin assembly protein gene (PICALM); ATP-bonding cassette, sub family A, member 7 (ABCA7); membrane spanning A4 (MSA4); CD2 associated protein (CD2AP); cluster of differentiation 33 (CD33); and ephrin receptor A1 (EPHA1) result in a genetic signature that might affect individual brain susceptibility to infection by the herpes virus family during aging, leading to neuronal loss, inflammation, and amyloid deposition.

Keywords: Alzheimer's disease, genetic background, GWA studies, herpes-virus

INTRODUCTION

Alzheimer's disease (AD) pathology is characterized by neuronal loss leading to brain atrophy and to a decrement of the cerebral metabolism. Major neuropathologic lesions are: (i) synapse and neuron loss; (ii) extracellular amyloid deposits and amyloid plaques, principally composed of amyloid-β (Aβ) peptide; (iii) intraneuronal accumulation of hyperphosphorylated tau proteins leading to neurofibrillary degeneration; (iv) reactive astrogliosis; and (v) brain inflammation. The incidence of AD is rising sharply and an increased number of elderly will ultimately be affected by the disease. Because of the urgency for effective preventive and therapeutic measures, extensive research has focused on pathogenetic mechanisms of the disease. Current views of AD pathogenetic mechanisms describe amyloid deposition and neuritic plaque formation as central mechanisms leading to neurodegeneration, cognitive impairment, and sporadic AD. Therefore, therapeutic approaches have focused on reducing amyloid load and plaque deposition or clearance of brain amyloid. However, a therapy is not already available.

Other mechanisms may be closely related with the etiology and pathogenesis of sporadic AD. This disease in fact is one of the most heritable common complex diseases with a heritability ranging 60–80%, as simplified by the association of the APOE gene with the

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disease, where the presence of 1 or 2 APOE4 alleles considerably increase the risk of AD. However, concordance rate for AD in monozygous twins is no higher than 61% and AD heritability decreases with increasing age [1]. Environmental risk factors are still largely unrevealed in AD, even if they may accumulate with advancing age and play the role of multiple triggers of the disease in the susceptible brain. Here, we discuss recently published genetic data from genome wide association (GWA) studies on several thousand AD patients and controls (CTR) [2, 3] showing that a limited number of genes were highly associated ($p > 10^{-5}$) with the disease. The effect of a single SNP or gene, with the exception of APOE, was small; therefore, the urgent challenge is to take into consideration genetic risk factors in the context of environmental risk factors or protective variables.

GWA DATA, THEIR INTERPRETATION, AND ENVIRONMENTAL FACTORS

One key question is: how can we make mechanistic sense of a collection of weak associations between SNPs and a diseases phenotype, i.e., AD? This question is perhaps the most difficult to solve and it stems from the heart of GWA studies focused upon common complex diseases. In fact, in a complex trait, as is the case in AD, several of the loci with weak effects might code for proteins that would interact in common pathways to yield a synergistic mechanism of action in AD pathogenesis. This is exactly the situation applicable to our previous GWA investigation and to other similar independent GWA studies confirming the association data in AD. In fact, in spite of the elevated numbers of patients and controls from AD GWA studies, each single SNP showed a modest OR for the diseases, usually <2.0. These findings are suggestive of the following considerations: 1) Interactions among different SNPs in diverse genes might be more informative than a single SNP. 2) None of these genes alone is causative for the diseases. 3) All described genes are however involved in different aspects of AD pathogenesis and/or clinical history. 4) Environmental factor(s) might trigger several of these genes. 5) Many of these genes upon activation by environmental factor(s) would turn on or influence other genes that would affect secondary pathogenetic mechanisms in the brain such as apoptosis, immune responses, cholesterol synthesis and transportation, and oxidative stress. Here we suggest that infective agents of CNS, such as viruses of the herpes family, are the probable link for all SNPs found associated with AD from recent GWA studies and the view presented here supports the notion of an infective etiology of sporadic AD.

SNPs ASSOCIATED WITH AD AND VIRUS INFECTIONS

The first set of genes was located in close vicinity of the APOE locus on chromosome 19 and consisted of the poliovirus receptor-related 2 or nectin-2 (NC-2), apolipoprotein E (APOE), the translocase of outer mitochondrial membrane 40 homolog (TOMM40), the glycoprotein carcinoembryonic antigen related cell adhesion molecule-16 (CEACAM-l 6), and B-cell/lymphoma-3 (Bcl-3) genes. Genes in the second set were located in different chromosomes: APOJ or clusterin on chromosome 8; the complement receptor l (CR-l) on chromosome 1, and C-type lectin domain family 16 member A (CLEC 16A) on chromosome 16. Polymorphic variations in each of these genes were individually associated with AD (P values ranging from 10^{-16} to 10^{-5}). We already discussed in another publication the relevance of these genes along with PICALM gene association for the virus susceptibility and AD pathogenesis [4] as summarized in Table 1.

Table 1
First set of genes GWA studies resulted associated with AD risk

Gene		Chromosome	GWA studies
CEACAM16	Carcinoembryonic antigen-related cell adhesion molecule 16	19	[2, 3]
BCL3	B-cell CLL/lymphoma 3	19	[2, 3]
PVRL2	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	19	[2, 3]
TOMM40	Translocase of outer mitochondrial membrane 40 homolog (yeast)	19	[2, 3]
APOE	Apolipoprotein E	19	[2, 3]
APOC1	Apolipoprotein C-1	19	[2, 3]
CLU	Clusterin	8	[2, 3]
CR1	Complement receptor 1	1	[2, 3]
CLEC 16A	C-type lectin domain family 16 member A	16	[2, 3]
PICALM	Phosphatidylinositol binding clathrin assembly protein	11	[2, 3]

Table 2
Second set of genes GWA studies resulted associated with AD risk

Gene		Chromosome	GWA studies	Functions
ABCA7	ATP-bonding cassette, sub family A, member 7	19	[5, 6]	[7–13]
MSA4	Membrane spanning A4	11	[5, 6]	[14–20]
CD2AP	CD2 associated protein	6	[5, 6]	[21–24]
CD33	Cluster of differentiation 33	19	[5, 6]	[25–30]
EPHA1	Ephrin receptor A1	7	[5, 6]	[31–34]

FURTHER GWA DATA SUPPORTING THE INFECTION HYPOTHESIS

A third new set of genes has emerged by a very recent GWA AD studies [5, 6] as summarized in Table 2. The association of these five genes with AD also appears to support the virus infection hypothesis in AD. This last group consisted of the following genes: ATP-bonding cassette, sub family A, member 7 (ABCA7), membrane spanning A4 (MSA4), CD2 associated protein (CD2AP), cluster of differentiation 33 (CD33) and ephrin receptor A1 (EPHA1), and here we suggest their potential relevance in virus infection and AD.

ABCA7 is highly expressed in the brain, especially values in hippocampal CA1 neurons [7] and microglia [8] and regulates the efflux of lipids from cells to lipoproteins. Moreover ABCA7 influences the quality of lipoprotein by interacting with APOA1 molecules especially in females, since this gene is involved in the assembly reaction of high density lipoprotein (HDL) [9] and controls heterogeneity of HDL [10]. It is known that certain viruses can circulate in biological fluids bound to lipoproteins; for instance, hepatitis C virus particles circulation is associated with plasma lipoproteins [11]. Therefore, the type of lipoproteins and lipids might influence virus transport to a given tissue and its circulation within the brain especially in women; incidence and prevalence of AD being higher in women. This gene also affects the efficiency of phagocytosis of apoptotic cells by monocyte cell lineage [12] and clearance of apoptotic virus infected cells [13]. Therefore, ABCA7 variants might also influence clearance of infected cells from the brain.

The MSA4 gene belongs to a genetic cluster located on chromosome 11 and encodes for the beta sub-unit of high affinity IgE receptor [14, 15]; this molecule is a component of an oligomeric cell surface complex involved in signal transduction in different cell lineages [16]. The MSA4 cognate protein has been involved in antiviral responses in human plasmocytoid or lung dendritic cells [17, 18]. Furthermore, CD23 or Fc-epsilon IIIR play a role in astrocyte inflammatory response during HIV-1 encephalitis [19] and HIV-1 infection induces an impaired regulation of the IgE Fc-epsilon RI network [20]. Therefore, this gene might influence virus entrance in neuronal cells and virus infectivity might in turn affect the expression of this membrane complex.

CD2AP gene is located on chromosome 6 and codes for a member of a novel family of scaffold/adaptor proteins, expressed on several cell types and regulates the actin cytoskeleton [21]. CD2AP plays an important role in antivirus defenses, since it regulates transportation and fusion of cytoplasmic granules in NK cells [22]. Moreover this molecule also affects selective activation of survival pathways and repression of apoptosis signaling by TGF-beta [23]. CD2AP might play multiple roles by regulating defense mechanisms against virus infectivity and cell sensitivity to apoptosis induced by the virus infection. Finally, CD2AP, by affecting early endosome morphology and traffic between early and late endosomes [24], might disturb AβPP metabolism and amyloid deposition.

CD33 gene is on chromosome 19 and codes for a member of the sialic-acidic-binding immunoglobulin like lectin or SIGLEC family that promotes cell-cell interactions and regulates immune functions of both innate and adaptive immunity [25]. Human cytomegalovirus latent infection induced the upregulation of the MCP-1 molecule in a restricted subset of CD33 positive myeloid progenitor cells and this mechanism may contribute to virus dissemination [26, 27]. Human herpes virus 7 also induced an upregulation of CD33 in cultured human cells [28]. Moreover, microarray analysis of blood mononuclear cells from HIV-1 positive patients on retroviral therapy showed an overexpression of CD33 molecule [29]. In liver

Kupffer cells infected by HCV overexpressed the CD33 molecule [30]. Once again one gene might affect multiple steps involved in herpes infectivity and individual susceptibility to virus infection.

EPAH1 is a member of the ephrin receptor subfamily of tyrosine-kinases and mediates cell and axon guidance, synaptic development and plasticity [31, 32]. This molecule is also implicated in apoptosis [33] and inflammatory response regulation [34]. EPAH1 might be implicated in antivirus resistance by affecting both apoptosis impairment induced by the virus infection, the efficiency of the host immune responses and synaptic plasticity of infected neurons.

In conclusion we argue that the concomitant presence of several SNPs in these genes in the same individual might represent a genetic signature of AD and further reinforce our hypothesis that such genetic trait predisposes to AD via complex and diverse mechanisms each contributing to the differential individual brain susceptibility to viral infections.

Evidence from other investigators showing HSV-1 infection in AD brains is on record [35–37]. It is of interest that the concomitant presence of the APOE 4 allele and vertical transmission of HSV-1 has been shown to confer a differential risk of brain infection and AD [38]. Moreover, APOE 4 deficient mice had significantly lower virus load in CNS than APOE 4 transgenic mice [39, 40]. Other studies also showed an association of HSV-1 with AD and influence of APOE allele [41–43] Reactivation of HSV-1 in the brain was also found in patients with familial AD who showed increased viral DNA and protein expression in cortical neurons [44]. HSV-1 has been also related to Down's syndrome, a condition at high risk for AD type dementia [45]. It is of interest that mothers of children with Down's syndrome showed increased serum HSV-2 antibody levels [46]. Moreover, HSV-1 induces the intracellular accumulation of Aβ in autophagic compartments of neuroblastoma cells [47] and in rat cortical neurons [48]. It has also been recently suggested that AD plaques and tangles might represent a cemetery of a partially unsuccessful immune response against herpes simplex infection [49]. It is important to keep in mind that herpes simplex glycoprotein B generated peptide fragments with high homology with Aβ peptide, forming fibrils and inducing neurotoxicity [50] and HSV-1 infection induced AβPP processing resulting in Aβ peptides formation in rat neuronal cells [51]. Moreover, findings showing that intracerebral infusion of AD brain extracts induced neurodegeneration in human AβPP transgenic mice is compatible with an infective etiology of dementia [52].

FURTHER SUPPORT TO VIRUS INVOLVEMENT IN THE DISEASE, THE OLFACTORY VECTOR HYPOTHESIS OF AD

As we already discussed, the cause(s) of AD is(are) still obscure. Olfactory dysfunction in the early history of the diseases is well documented [53]. The presence of smell loss and olfactory bulb pathology in the early stages of AD together with the evidence that airborne xenobiotics, representing AD risk factors, can enter the brain via the olfactory mucosa has led to the hypothesis that the disease may be caused or activated by agents that enter the brain via the nose. Moreover, the olfactory nerve is uniquely vulnerable to virus penetration. In fact, the dendritic knobs and protruding cilia of millions of olfactory receptor cells provide an exposed surface area of about 23 cm^2. These cells are widely distributed throughout the rostral nasal cavity, embedded in a specialized neuroepithelium and are first order neurons projecting axons directly to the brain. It is of historical relevance to note that olfactory receptor cells were the major route of entry for poliomyelitis viruses into the brain. HSV-1 placed intranasally in mice is detected in the olfactory bulbs after several days; thereafter, it infects cholinergic neurons of several brain regions [54]. Approximately 90% of AD patients in the early stage of the disease exhibits olfactory dysfunction and longitudinal studies suggest that olfactory deficit in AD precedes cognitive impairment by several years [55]. Moreover, tau-related pathology within olfactory bulb and anterior olfactory nucleus was detected [56]. Virus may access the brain via olfactory bulb and become latent in several brain areas connected to the olfactory nucleus. Therefore, the investigation of these target areas may give crucial information regarding the relevance of virus infection, latency and transmission of virus vector to the brain cortex.

SNPs IN OTHER GENES REGULATING INFLAMMATORY RESPONSES SIGNALED BY CASE/CONTROL INVESTIGATIONS MAY ALSO PLAY A ROLE BY INFLUENCING VIRUS LATENCY AND INFECTION SUSCEPTIBILITY

Our previous work showed that alpha-1-antichymotrypsin (ACT), a protease inhibitor and acute phase protein, was elevated in plasma, cerebrospinal fluid, and brains from AD patients [57–60]. ACT plasma levels correlated with cognitive decline [57, 60] and SNP

in the promoter gene of the ACT gene was associated with increased risk of AD, fast cognitive deterioration and elevated levels of plasma levels of the cognate protein [58]. Elevated plasma ACT has also been found in non demented elderly with decreased cognitive performances [61]. It of interest that elevated serum ACT from HIV-1 positive women has been found and its levels correlated with the viral load [62]. Moreover, ACT containing globules within hepatocytes in patients with chronic hepatitis C and cirrhosis have also been reported [63]. Therefore, data from ACT gene association with AD and increased levels of ACT blood protein with cognitive decline and the disease progression might be compatible with an infective etiology of dementia.

Our previous investigations also showed that SNPs in the promoter region of several genes controlling for different cytokines synthesis and release, such as interleukim-1α (IL-1α), IL-1β, IL-6, IL-10, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were differentially associated with the risk of AD [64]. These results may also be explained by the virus infection hypothesis, since individual differential ability to mount an effective immune response can influence the control of the virus latency and individual susceptibility to virus re-infection both in peripheral tissues and brain. Finally, we and other authors also showed an association of SNP in the promoter region of the VEGF gene with an increased AD risk [65–67]. These findings suggested that critical factors such as VEGF that are implicated in neo-angiogenesis, neurogenesis, and glia activation in adult brain, might also influence the clinical manifestation of cognitive impairment and AD. Impairment of neurovascular mechanisms leading to brain hypoperfusion, vessel regression, and neurovascular inflammation have indeed been suggested in AD, and micro-vascular pathology are frequent neuropathology features of the AD brain [68, 69]. HHV-6 during latency expresses the U94/rep latency associated gene and it has been recently shown that U94/rep protein inhibited the formation of *in vitro*-like capillary structures, the migration of endothelial cells and *in vivo* angiogenesis [70]. Finally, VEGF in mammals affects both angiogenesis and neurogenesis in the hippocampus [71]. Therefore, virus infection of brain vessels might impair angiogenesis and neurogenesis, ultimately affecting neuronal repair and survival in critical brain areas such as the hippocampus and contributing to neurodegeneration processes in individuals with low intrinsic capacity to produce angiogenic factors such as VEGF. This notion is indirectly supported by our recent publication showing a partial overlapping of the genetic background between AD and a classical vascular disease such as acute myocardial infarction [72].

The above mentioned SNPs did not show up in the recent GWA studies based upon highly statistically association with the disease, and we might conclude that they are secondary linked to AD. However, it is important to note that even SNP in genes such as ABCA7, MSA4, CD2Ap, CD33, and EPAH1 individually appear to play a limited role in AD pathogenesis, since their OR values are between 1.1 and 1.4. Therefore, it is unlikely that they are causative for the disease.

However, the concomitant presence of several SNPs in many of the above discussed genes in the same individual by impairing body resistance to microorganism infection and/or favoring virus latency and re-infection in the brain over a time interval of several years might results in a genetic signature predisposing to AD.

CONCLUSIONS

Viruses of the herpes family are among the most probable pathogen candidates to CNS neurodegeneration in old age, because of their well known ability to escape peripheral immune responses by invading neurons. The relevance of herpes virus in aging is supported by a recent investigation showing that during aging a substantial proportion of peripheral CD8 T cytotoxic cells of elderly have been found to be directed against EBV and CMV [73].

Moreover, the aged immune system may be no longer able to control virus reactivation [74]. Therefore, viral infection becomes chronic in a large proportion of the elderly. Finally highly pathogenic H5N1 influenza virus has been shown to enter the brain and induce neuroinflammation and neurodegeneration, and this virus has been suggested to be involved in Parkinson disease [75]. It is important to note that up to now most of the investigations have shown an association of HSV1 with AD [35–43]. However, CMV and HSV-2 might play a role in cognitive decline during aging or dementia in Down syndrome patients [46, 76]. Moreover HHV6 was found in brain specimens of control elderly and AD patients although HHV-6 did not appear to be specifically associated with dementia [77]. Therefore, viruses may play multiple and unsuspected role in neurodegeneration of CNS and be the initial hit starting a vicious cycle leading after several years to irreversible brain decline. A flow chart representing the complex interplay among epidemiological, genetic, virus, and inflammatory factors inducing sub-clinical

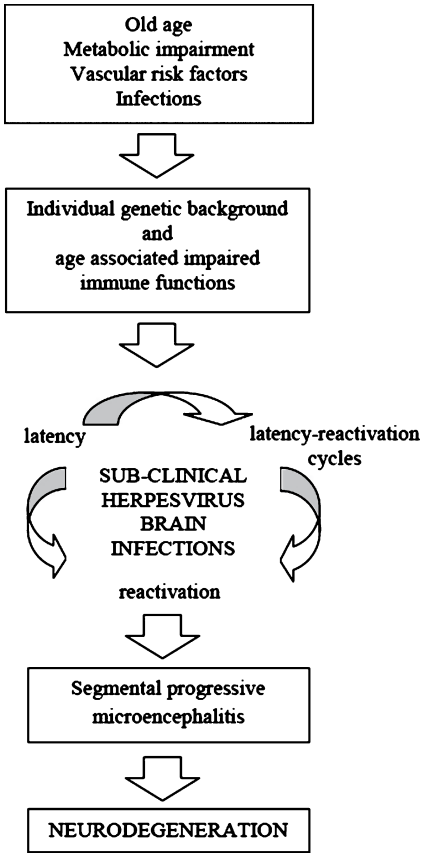

Fig. 1. Complex relationship among risk factors related to brain infections during aging leading to AD.

and chronic neuronal loss is reported in Fig. 1. With advancing age an impaired immune system might facilitate virus reactivation in the brain, especially in those subjects showing the suggested genetic signature. It is important to stress that studies on HLA polymorphisms association appear to support a viral infection involvement in AD pathogenesis [78].

Latent or chronic viral infection has been indeed found to correlate with the rate of cognitive decline in the Sacramento Area Latino Study on Aging [73]. Therefore, brain infection by reactivated latent viruses might induce progressive neuronal loss, astroglia activation, and, by impairing AβPP transport along the axons [36], AβPP misappropriate metabolism and amyloid deposition.

The concomitant presence of several SNPs in many of the above discussed genes in the same individual might results in a genetic signature predisposing to AD, since they contribute to facilitate virus entrance, and latency and impair mechanisms of defense and resistance to microorganism infection.

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Section 9
Bacterial lipopolysaccharide and pathogen free conditions related to Alzheimer's disease

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Bacterial Lipopolysaccharide (LPS) and Alzheimer's Disease

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Abstract. Data found in literature have reported that bacterial endotoxins may be involved in the inflammatory and pathological processes associated with amyloidosis and Alzheimer's disease (AD). In fact, it has been observed that the chronic infusion of the bacterial lipopolysaccharide (LPS), the outer cell wall component of Gram negative bacteria, into the fourth ventricle of rats reproduces many of the inflammatory and pathological features seen in the brain of AD patients. In this context, a key player in the pathogenesis of AD is the amyloid-β peptide (Aβ) that is capable of aggregating in fibrils that represent the main component of amyloid plaques. These deposits that accumulate among brain cells are indeed one of the hallmarks of AD. This aggregation in fibrils seems to correlate with Aβ toxic effects. However, recent data have shown that amyloid fibril formation not only results in toxic aggregates but also provides biologically functional molecules; such amyloids have been identified on the surface of fungi and bacteria. The aim of this work was to get a better insight on the influence of bacterial endotoxins on Aβ fibrillogenesis; factors that influence fibril formation may be important for Aβ toxic potential. Following 3 days of incubation at 37°C, Aβ was organized in compact fibrils and the *in vitro* Aβ fibrillogenesis was potentiated by the Escherichia coli endotoxin. This suggests the importance of infectious events in the pathogenesis of Alzheimer's disease and proposes a new aspect related to the putative pathological factors that can be implicated in the mechanisms involved in $A\beta_{25\text{-}35}$ fibrillogenesis.

Keywords: Amyloid-β (Aβ), lipopolysaccharide (LPS), *Escherichia coli (E. coli)*, Alzheimer's disease (AD), transmission electron microscopy (TEM)

INTRODUCTION

Amyloid-β (Aβ) protein fragment is the major component of senile plaques found in the brains of patients with Alzheimer's disease (AD). This peptide is cleaved from a larger protein called amyloid-β protein precursor (AβPP) that is an ubiquitously expressed transmembrane glycoprotein. In plasma and cerebrospinal fluid, amyloid-protein exists primarily as a soluble peptide of 40–42 residues, while in senile plaques Aβ forms amyloid fibrils mediating neurotoxic activity [1]. The amyloid fibril ultrastructure is characterized by fibers of 7–12 nm in diameter and of indeterminate length, since fibril assembly is associated with a β-sheet conformation as observed by x-ray diffraction pattern [2–5]. These fibrils are different in the various pathologies, but exhibit several common physico-chemical features: fibrillar morphology, predominantly β-sheet secondary structure, affinity for binding thioflavin S, apple-green birefringence on Congo Red, very high stability and protease-resistance [2]. Amyloidosis of the secondary type occurs as an occasional consequence of chronic inflammatory processes; this process is characterized by the deposition of extracellular fibrils composed of Amyloid A protein (AA), and a serum amyloid A protein (SAA) modified by proteolitic removal of the C-terminal aminoacids [6].

The causative agents of these highly complex diseases, which are often the result of several combined genetic and environmental factors, are still unknown and the molecular basis underlying their pathogenesis has yet to be fully clarified [7]. Several studies

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on experimentally induced amyloidosis performed in domestic ducks [8], hamsters [9], rabbits [10], and mice [11] have shown that different bacteria, following repeated inoculations, may produce histologic amyloidotic changes in spleen, liver, and kidney that resemble the chronic lesions seen in man [10], and that are probably due to the continuous septic conditions. The study by Bowery [12] showed the neurodegenerative effects produced by tetanus toxin in rats, and a significant emerging body of literature suggests the possibility that CNS infections may play a cofactorial role in inducing neurodegenerative diseases [13]. In fact, it has been observed that chronic infusion of bacterial lipopolysaccharide (LPS), the outer cell wall component of Gram negative bacteria into the fourth ventricle of rats reproduces many of the inflammatory and pathological features seen in the brain of Alzheimer's disease (AD) patients [6].

INFECTIOUS AGENTS

In studies on humans, several authors reported the existence of an association between *Chlamydia pneumoniae*, an obligate intracellular respiratory pathogen, and AD [14–18]. The cerebrospinal fluid and cerebral cortex of patients with general paresis provided evidence that Spirochaetes (Borrelia burgdorferi, Treponema pallidum) [17, 19] are responsible for slowly progressive dementia, cortical atrophy and local amyloidosis. Spirochetes form plaque-like masses and disseminate as individual filaments which are identical to senile plaques and curly fibres [17]. In addition, they are all linked to periodontal polybacterial disorders, which are primarily caused by Gram-negative bacteria [20–22].

The causative agent of stomach ulcers, Helicobacter pylori (H. pylori) [23], has also been suggested to be associated with AD [24, 25]; serum IgG and IgA antibodies against H. pylori occurred in a higher percentage in the group of AD patients [26].

The infectious agents involved in the pathogenesis of AD are also linked to atherosclerosis, cardio- and cerebrovascular disorders [27–30], chronic lung diseases [31], inflammatory bowel diseases, and various neurological and neuropsychiatric disorders [32–35]. Epidemiological studies have confirmed these data [36].

An association with diphtheria toxin would be consistent with the observations that the bacteria associated with the toxin, *Corynebacterium diphtheria*, is often found in the nasopharynx and an early symptom of AD is the loss of smell with a disease progression from the entorhinal cortex to the hippocampus and the neocortical areas [37].

As in syphilis, systemic infection and inflammation precede the development of dementia by years or decades [17].

Microrganisms can use biosurfactants (i.e. LPS endotoxin) to regulate their cell surface properties to attach or detach from surfaces. Following recent observations amyloids are assembled at microbial surfaces. Their role in bacterial and fungal invasion has already been discussed [38, 39]. By activating host proteases involved in the haemostatic system, microbial amyloids have been implicated in colonization of the host and might contribute to complications during sepsis. These structures are involved in the attachment of the bacteria to inert solid surfaces and also function in biofilm formation; curli and tafi fibrils also function in bacterial virulence [17].

Inflammatory mediators

During chronic exposure, bacteria and bacterial debris accumulate in infected host tissues, sustaining chronic inflammation and slow, progressive cell damage [6, 40–42]. They are not only inflammatory cytokine inducers and activators of complement pathways, but they also affect vascular permeability [43], induce nitric oxide and free radicals, inhibit DNA synthesis, and cause apoptosis and cellular damage [44].

Aβ oligomers/fibrils induce intracellular calcium deregulation that leads to apoptosis through mitochondrial dysfunction by direct interaction with isolated mitochondria or by indirect association with the neuronal membrane [45]. Disruption of intracellular homeostasis of Ca2+ by channel opening has been extensively proposed as a mechanism of Aβ neurotoxicity [46, 47].

The long-term effects of persistent or lifelong repeated infections may differ in different hosts, according to their general health, pharmacological treatments, genetic background (Apolipoprotein E, APOE $\varepsilon 4$ enhances the expression of inflammatory mediators) [48, 49], or concurrent diseases. Long-term use of anti-inflammatory drugs alone might weaken the elimination of pathogens and facilitate their evasion, survival and slow, progressive proliferation. Combined antibiotic, antiviral and anti-inflammatory therapy is suggested as the treatment of choice [17].

$A\beta_{25\text{-}35}$ fragment

Within this context the *in vitro* interaction between the $A\beta_{25\text{-}35}$ fragment and the Escherichia coli endotoxin at different concentrations was observed by transmission electron microscopy, with the purpose to gain a better insight on their time-dependent morphology, physiochemical organization and the possible influence of the LPS endotoxin on Aβ fibrillization. $A\beta_{25\text{-}35}$ fragment was chosen because this short peptide has been proposed to be a functional domain of Aβ responsible for its neurotoxic properties [50, 51]. In addition, Aβ fragment was also exposed to different concentrations of a suspension of viable intact *E. coli* ATCC 25922 cells to verify if the effect observed with LPS was reproducible with *E. coli*.

LPS experiments

The LPS stock suspension from *E. coli* serotype 0128:BI2 was prepared at the concentration of 3 mg/ml. Aliquots of 0.1μg/ml, 1 μg/ml and 10μg/ml respectively were combined with $A\beta_{25\text{-}35}$ fragment, dissolved in distilled water (final concentration 0.5μg/ml) and observed by transmission electron microscopy (TEM) after three days incubation at 37°C. Additional observations were performed after keeping the samples for 2.5 weeks at room temperature.

Control experiments: LPS at the concentration of 100 μg and $A\beta_{25\text{-}35}$ fragment (final concentration 0.5μg/ml) were independently observed by TEM after three days at 37°C. Additional observations were performed after keeping the samples for 2.5 weeks at room temperature. For each condition, four independent experiments were performed.

Escherichia coli experiments

E. coli ATCC 25922 was grown overnight in Tryptone Soya broth, then centrifugated and finally washed three times in PBS (pH 7.3). The obtained stock solution was at the concentration of $2\text{–}4 \times 10^7$ CFU/ml. Aliquots were prepared from this stock solution, each containing viable cells of about 10/100/1000 CFU. Aβ fragment 25–35 was added to each suspension to obtain a final concentration of 0.5 μg/ml. Samples were incubated at 37°C for three days and observed by TEM. Additional observations were performed keeping the samples at room temperature for 2.5 weeks.

Control experiments

E. coli, prepared in distilled water at the concentration of 10/100/1000 CFU, and $A\beta_{25\text{-}35}$ fragment (prepared as previously described) were independently observed by TEM after three days at 37°C and after 2.5 weeks at room temperature.

For each condition, four independent experiments were performed.

Transmission electron microscopy

Samples were prepared with the Negative Staining technique by floating small aliquots (20μl) of aqueous suspension on formvar/carbon coated glow-discharged grids for 2 minutes; then they were air dried and stained with 2% uranyl acetate for 2-3 minutes. The advantage in using uranyl acetate is that the dried specimen grids remains stable over a period of weeks. The observations, at transmission electron microscopy, were carried out at several positions across each grid to avoid biased selections.

IN VITRO AMPHIPATIC ORGANIZATION OF $A\beta_{25\text{-}35}$ AND LPS

Biochemical *in vitro* studies on Aβ-peptide have established that long incubation times promote amyloid assembly [5, 52]. In these experiments we observed, after 3 days incubation at 37°C a spontaneous *in vitro* fibrillogenesis of $A\beta_{25\text{-}35}$ peptide in short smooth amyloid fibrils (Fig. 1A). Consistent with the reported Aβ surfactant properties, the same samples were observed after 2 weeks at room temperature: long smooth branching fibrils appeared decorated by micelles (Fig. 1B). No significant differences were noticed in the samples observed after 2.5 weeks at room temperature.

Bacterial LPS also displays surfactant properties having an hydrophobic alkylic chain and an anionic headgroup, and it may aggregate into different physical structures such as micelles or bilayers. LPS in aqueous solution was incubated for 3 days at 37°C. Micellar particles of different sizes were detectable (Fig. 2A and inset). Figure 2B shows a large micelle formed by endotoxin after 2 weeks at room temperature; free micelles are detectable on the background. The empty space (Fig. 2B) was probably formerly occupied by small micelles that generated the large one. We also observed membrane bilayers formed by the endotoxin (not shown).

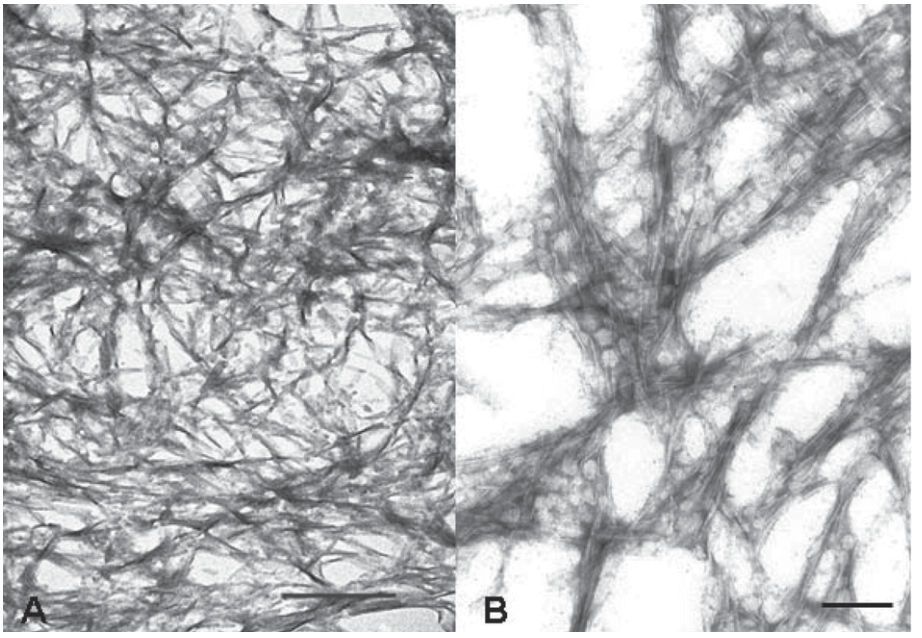

Fig. 1. *TEM images of Aβ25-35 peptide*. Panel A: after 3 days incubation at 37°C, spontaneous *in vitro* fibrillogenesis of $A\beta_{25-35}$ peptide in short smooth amyloid fibrils of different length. Bar = 500 nm. At time zero, controls contained no fibrils. Panel B: after 2 weeks at room temperature, long amyloid fibrils with regular helical twist are detectable. Little micelles are widely spread among them. Bar = 100 nm.

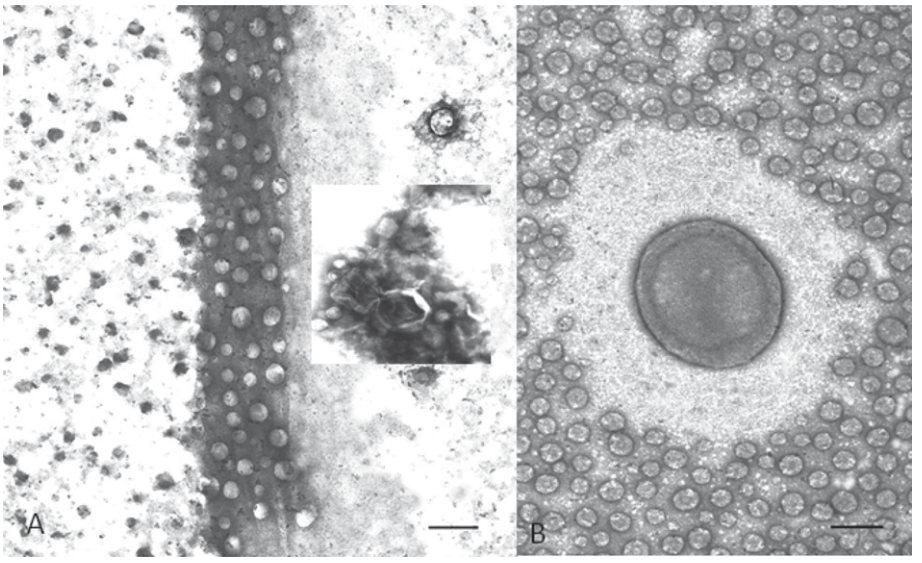

Fig. 2. *TEM images of LPS*. Panel A: after 3 days incubation at 37°C, LPS is aggregated to form micellar particles. Bar = 200 nm. Panel B: after 2 weeks at room temperature a large micelle is clearly detectable in the centre of the micrograph. There are free micelles in the background. Bar = 200 nm.

Time-dependent interaction between LPS and $A\beta_{25-35}$ fragment

In the second part of the study, Aβ fragment was incubated for 3 days at 37°C in the presence of different amounts of LPS. The aim of these experiments was to observe if there was an interaction between LPS and Aβ, at molecular level, on the basis of their common surfactant properties and considering that detergents and fatty acids are able to form micelles at

active concentrations [53]. We observed that LPS is able to accelerate the Aβ peptide assembly increasing its toxicity. In Fig. 3A, LPS is present at 0.1 μ g/ml: after 3 days incubation at 37°C, long smooth twisted fibrils are coated with a thin layer of LPS; some fibrils are extending from the nucleation centres. In Fig. 3B, small micelles are detectable in the background. After 2 weeks (Fig. 3C) at room temperature Aβ fibrils seem to be linked or in close association with LPS (arrow). Figure 4A (LPS 0,1μ g/ml) shows long smooth branched 10–12 nm helical fibrils; a considerable potentiation of Aβ fibrillogenesis has occurred. Fibril binding to micellar particles is more marked in these samples when observed after 2.5 weeks at room

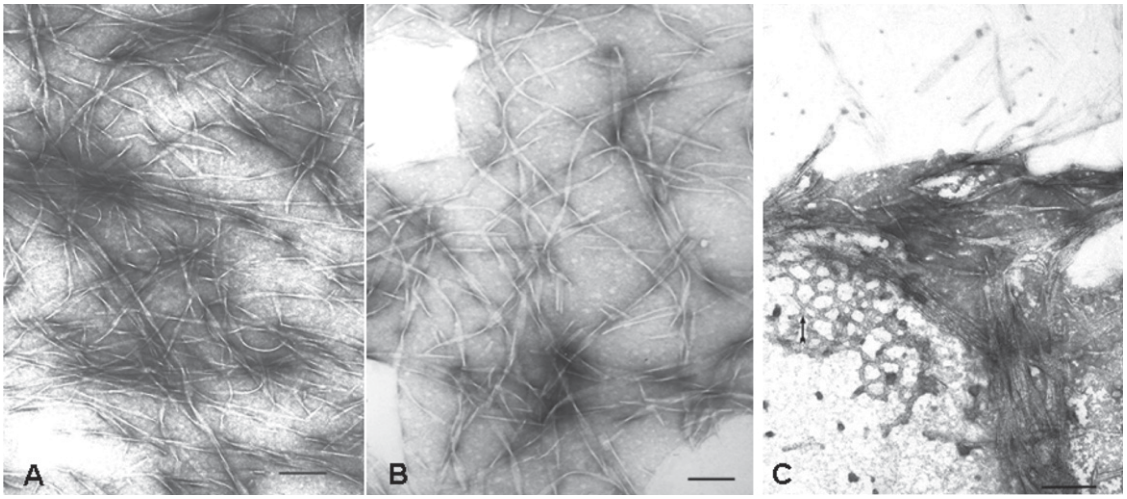

Fig. 3. *Time-dependent interaction between $A\beta_{25\text{-}35}$ fragment and LPS*. Panel A: after 3 days of incubation at 37°C, Aβ is organized into structured twisted fibrils (arrow) covered by a thin layer of LPS. Nuclear centres are also detectable. Bar = 200 nm Panel B: small micelles are detectable among fibrils in the thin layer of LPS (arrows). Bar = 200 nm. Panel C: after 2 weeks at room temperature, there is a close association between fibrils and LPS (arrow). Scattered filaments in the background. Bar = 200 nm.

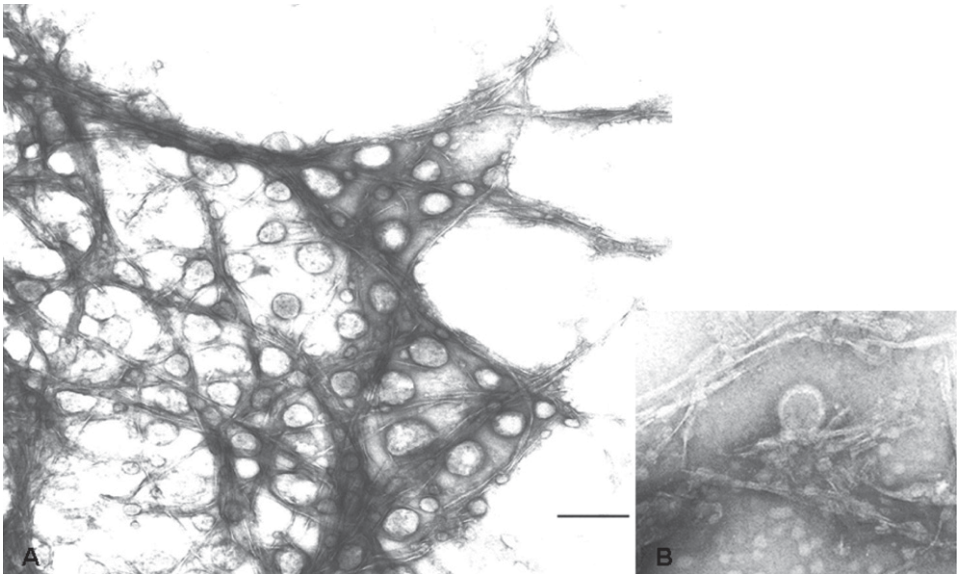

Fig. 4. *Time-dependent interaction between $A\beta_{25\text{-}35}$* fragment and LPS. Panel A: after 2.5 weeks at room temperature, Aβ fribrillogenesis was enhanced in presence of LPS. Long flexuous branched fibrils binding the micelle are detectable. Bar = 200 nm Panel B shows the binding of short fibrils to the surface of a micelle Bar = 200 nm.

temperature. Panel B shows the binding of short fibrils to the surface of a micelle. After 3 weeks, long helical fibrils were visible in close association with micelles (Fig. 5 arrowhead). In the background, older micelles are about to release amorphous aggregate and protofilaments (arrow). Figure 6 shows a network of helical fibrils of different length; some fibrils are extending from a nucleation centre. No other micelles are clearly detectable (arrow). The features of these fibrils are completely different from those obtained from the $A\beta_{25\text{-}35}$ fragment incubated alone (Fig. 1); no other micelles are clearly detectable. The above results suggest that LPS constitutes an important cofactor in Aβ fibrillogenesis. The incorporation of LPS occurs at an early stage of Aβ aggregation acting as a nucleation factor or seed; then it acts in the elongation of the amyloid fibrils.

These experiments were performed in a time-dependent manner and, as mentioned, the images shown here are at the lowest LPS concentration (0.1μ g/ml). We also tested higher amounts of LPS in the presence of $A\beta_{25\text{-}35}$ fragment. At 1μ g/ml concentration we obtained similar results at all of the times investigated, while at 10μ g/ml LPS concentration it was difficult to get good quality images, especially after 2 weeks at room temperature, because the samples were too electron-dense for observation under the electron microscope. This indicates that the process is also concentration-dependent, as previously reported [5]. The samples here described were positive for apple-green birefringence of Congo red staining characteristics of beta-sheet rich fibrils (not shown).

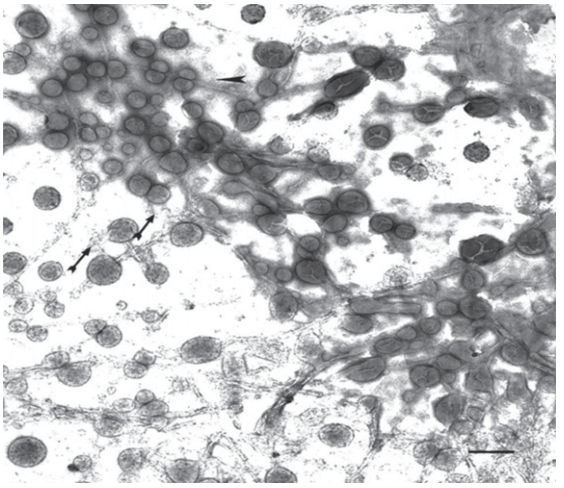

Fig. 5. *Time-dependent interaction between $A\beta_{25\text{-}35}$* fragment and LPS. Helical fibrils binding to the micelles is more marked (arrowhead). This indicates a direct affinity at molecular level; some older micellar particles are releasing aggregate from the surface (arrow). Bar = 200 nm.

Interaction between E. coli and $A\beta_{25\text{-}35}$ fragment

Aβ was combined with a viable intact cell suspension of *E. coli* ATCC 25922 at different concentrations. We made observations for all the concentrations tested and times investigated. Figure 7A shows the interaction between *E. coli* (10 CFU) and Aβ after 3 days incubation at 37°C. Short irregular scattered filaments different from the helical fibrils observed in presence of LPS, were closely in contact with the bacterial wall. Soft amorphous aggregate and Aβ short fibrils were linked and covered the bacterial wall (Fig. 7B). It is apparent that the bacterial wall has affinity for $A\beta_{25\text{-}35}$. Due to the negative staining, a fast procedure to screen samples with transmission electron microscopy, it was not possible to clearly distinguish the bacterial wall because the samples were not subjected to fixation and dehydration processes.

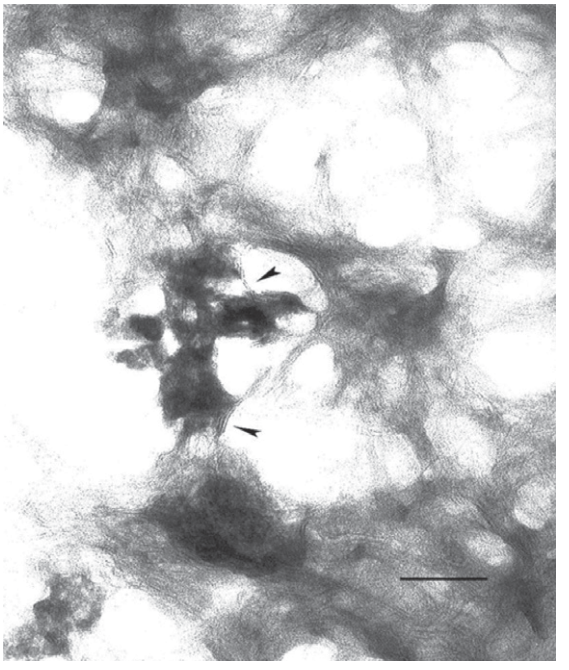

Fig. 6. *Time-dependent interaction between $A\beta_{25\text{-}35}$ fragment and LPS*. Aβ fibrillogenesis has been potentiated and the picture shows 10–12 nm diameter amyloid fibrils of different length. Some fibrils are extending from a nucleation center. The features of these fibrils are completely different from $A\beta_{25\text{-}35}$ fibrils incubated alone; no other micelles are clearly detectable (arrowhead). Bar = 200 nm.

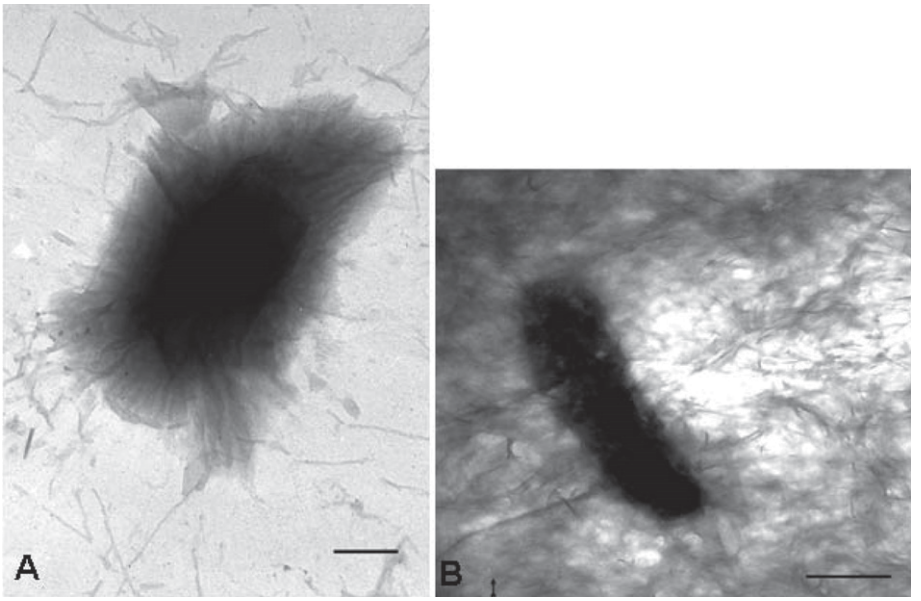

Fig. 7. *Interaction between $A\beta_{25\text{-}35}$ fragment and viable intact E. coli.* Panel A: after 3 days incubation at 37°C, a few short irregular filaments are closely in contact with the bacterial wall; in the background there are scattered filaments. Bar = 500 nm. Panel B: amorphous aggregate and Aβ thin fibrils are linked to the bacterial wall. Bar = 500 nm.

Amphipatic molecules and amyloid fibrils

Aβ displays surfactant properties, consistent with its ability to form micelles in solution. In fact, like detergents, Aβ peptide lowers the surface tension of water [5] and its folded structure is linearly amphipatic, since one end is polar and the other end is non-polar. Critical micelle concentration (CMC) is defined as the concentration of detergents above which micelles are spontaneously formed. In this context, Lomakin et al. [50, 52] proposed a model for Aβ according to which the amyloid peptide, at low pH, forms micelles above a critical concentration (Co>cmc, where Co is the peptide concentration). At this critical concentration, fibril growth becomes constant and it is independent from the initial peptide levels. In addition, since the fibrillogenesis process requires a nucleation step and micelles are regions of high peptide concentration, they can act as sites for the nucleation of Aβ fibrils that can successively grow by irreversible binding of Aβ monomers to fibril ends [52, 54]. It has also been reported that the incorporation of the surfactant n-dodecylhexaoxyethylene glycol monoether ($C_{12}E_6$) into Aβ micelles suppressed their ability to generate nuclei of fibrils to the point that heterogeneous nucleation dominated the nucleation process. This is the condition where Co<cmc, and the nucleation mainly occurs on non-Aβ seeds, the resulting fibrils are indistinguishable from those nucleated through micelles [52, 55]. These results suggested that the LPS was acting through two possible mechanisms. First, it might increase the seeds necessary for the nucleation step and second, it may stimulate fibril elongation without a concomitant incorporation in growing filaments [53].

The glycoprotein B (gB) of herpes simplex virus (HSV-1) has a highly homologous sequence to a fragment of Aβ [56]; synthetic peptides derived from this region accelerate fibrillar aggregation of Aβ *in vitro*. They can self-assemble into fibrils, which are ultrastructurally indistinguishable from Aβ and are neurotoxic at a similar dose to Aβ. It has been proposed that HSV-1 might act as a 'seed' for senile plaque formation [57].

Antimicrobial activity

Aβ has the capacity to associate with lipid bilayers (Fig. 8) also of bacterial cell membranes and to exert antimicrobial activity by membrane permeabilization and by alteration of calcium homeostasis [58, 59]. The bacterial membrane stain positive for Aβ following incubation with the peptide. This is consistent with a mechanism that involves association with microbial lipid bilayers [59]. An infectious ori-

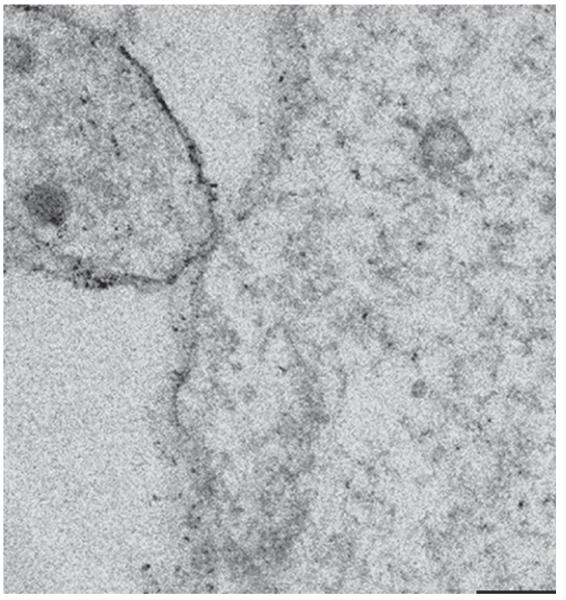

Fig. 8. SH-SY5Y cells with A-beta 1–42 after 48 h incubation at 37°C. Aβ has the capacity to associate with lipid bilayer (black spots) and to exert antimicrobial activity. Bar = 200 nm.

gin of AD is in harmony with recent observations showing that Aβ belongs to the group of antimicrobial peptides (AMPs), which are potent, broad spectrum bactericides targeting Gram-negative and Gram-positive bacteria, enveloped viruses, fungi and protozoans [59]. Identification of Aβ as an AMP raises the possibility that host cell citotoxicity, or at least a component of this activity, may also have a role in innate immunity [59–61].

ALZHEIMER'S DISEASE

More than one century ago Alzheimer [62] discussed the possibility that microorganisms could have a role in the formation of senile plaques; several authors considered that a slow-acting infectious agent, acquired at an early age and requiring decades to become active, might be involved in AD [17, 63, 64]. In AD patients, Aβ preferentially accumulates at brain level, however, although the blood brain barrier (BBB) is compromised in AD, the mechanism by which bacteria or infected cells may cross the BBB remains to be elucidated. Presumably bacterial pathogens breach the BBB and enter the CNS through paracellular, transcellular mechanisms or by inducing injury to the endothelium, thus resulting in BBB damage [65]. However, some pathogens are able to disrupt the BBB and cross directly into the CSF through the porous capillaries of the choroid plexus, thus passing into the brain and provoking severe haemorrhagic encephalitis. Infectious agents can reach the CNS by either crossing the blood brain barrier (haematogen route) or by being transported by axons of cranial nerve neurons [7].

AChE as chaperon

Acetylcholinesterase (AChE) plays a crucial role in the rapid hydrolysis of the neurotransmitter acetylcholine in the central and peripheral nervous system and might also participate in non-cholinergic mechanisms related to neurodegenerative diseases [66]. Inestrosa et al. have shown that in addition to its role in cholinergic synapses, AChE was able to accelerate the assembly of $A\beta_{1\text{-}40}$ into Alzheimer's fibrils by decreasing the lag phase of the peptide aggregation, suggesting a role of AChE as a chaperone for Aβ1-40 assembly into oligomers of a high structural complexity [66]. It has been shown [67, 68] that the level of an amphiphilic monomeric form of AChE is increased in the brain of transgenic mice which produce the human Aβ protein [67] and in the brain and cerebrospinal fluid (CSF) of rats which received intracerebral-ventricular injections of Aβ peptide. The capacity of AChE to promote assembly of Aβ into amyloid filament resides in the molecular structure of its monomer [69].

LPS: Chemical composition

Data found in literature on LPS show that the lipid A moiety from different bacteria has a very similar chemical composition and is responsible for the endotoxic effects at very low doses, which can include metabolic, circulatory and immunological effects [70]. In *E. coli* lipid A consists of glucosamine disaccharide, two phosphates and six acyl groups presenting a long alkylic chain. In this context, the ability of saturated fatty acids to lower the surface and the interfacial tension is indeed correlated to the presence of an alkylic chain in the range of 12–14 carbons. It has been suggested that emulsifiers, such as LPS, are virulence factors and their production by pathogens occur when the cell density is high enough to cause a localized attack on the host [71].

Infectious processes

The Cohen hypothesis on peripheral amyloid formation suggests that chronic stimulation of the

reticulo-endothelial cells leads to amyloid production [72]. Infectious processes could already begin in early childhood and only become manifest in old age; immunological imbalance processes could then gradually develop when defence mechanisms begin to deteriorate as a result of increasing age and accompanying diseases [73]. In this context, peripheral amyloidosis has been suggested as the result of an imbalance of the immune system due to an exaggerated antigenic stimulation [7], because disturbances of the immune system and changes at the microcirculation level may play an important role in the development of amyloid fibrils [7]. The outcome of infection is determined by the genetic predisposition of the patient, by the virulence and biology of the infecting agent and by various environmental factors; pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), recognise unique structures of invading microorganisms [74]; patients with genetic defects related to signalling pathways activated by TLRs frequently suffer from severe recurrent infection [17, 75, 76]. Aβ assembly is enhanced through the association with the so called "pathological chaperones", such as α-1 antichymotrypsin and apolipoprotein E [77, 78], LPS may also be considered as a pathological chaperone.

Therapeutic drugs

A major issue in AD research is to find some new therapeutic drugs which decrease Aβ aggregation[79]. From a therapeutic point of view the major question is whether pharmacological inhibition of inflammation pathways will be able to safely reverse or slow the course of disease. An infectious origin might give a comprehensive explanation of the common cellular and molecular mechanisms, inflammatory processes involved in chronic inflammatory disorders and in AD [17]. A prospective epidemiological study on populations that have suffered from persistent or lifelong repeated infections would provide evidence about the fact that there is an association between infections and the incidence of Alzheimer's disease; chronic infection caused by one or more infectious agents should be considered a risk factor for sporadic AD.

Long-term use of anti-inflammatory drugs alone might weaken the elimination of pathogens; prevention and early treatment with an adequate combined antibiotic and anti-inflammatory therapy is suggested as the treatment of choice and may limit the number of induced amyloid plaques *in vivo* and delay or prevent the future development of AD.

CONCLUSIONS

The present research demonstrate that LPS acts *in vitro* as an Aβ fibrillogenesis promoter, in a time-dependent manner, possibly through an heterogeneous nucleation mechanism or as a catalyst promoting Aβ aggregation without a concomitant incorporation in the growing filaments. This is very important considering that factors that facilitate Aβ fibrillogenesis may be critical for Aβ toxicity [80]. The direct binding of the Aβ fibrils to LPS micellar particles indicates a direct affinity at the molecular level. Moreover, the experiments performed with *E. coli* viable suspensions suggest the importance of infectious events in the pathogenesis of Alzheimer's disease [81–85] and open an additional perspective on the Aβ fibrillogenesis process. The next step of research could be an *in vivo* experiment: intra-hippocampal injections with aggregate $A\beta_{25-35}$ can model aspects of Alzheimer's disease (AD) in rats; the interaction between the $A\beta_{25-35}$ fragment and the *E. coli* endotoxin at different concentrations, could suggest a more detailed understanding of the molecular mechanisms underlying pathogen-mediated neuronal damage and may propose new preventive and/or therapeutic strategies aimed at counteracting the progression of AD.

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Pathogen Free Conditions Slow the Onset of Neurodegeneration in a Mouse Model of Nerve Growth Factor Deprivation

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Abstract. Several studies suggest that systemic infection occurring during ageing and chronic neurodegenerative diseases can evoke an exaggerated immune response that contributes to the progression of neurodegeneration and cognitive decline. However, studies directly addressing the relationship between microbial environment and the onset of neurodegeneration in Alzheimer's disease (AD) animal models are lacking. Here we show that the onset of neurodegeneration that transgenic mice develop when raised in conventional husbandry slows down when raising anti-Nerve Growth Factor transgenic mice in a murine pathogen free condition.

Keywords: Nerve Growth Factor, deprivation, amyloid-β, phosphorylated tau, microglia, systemic inflammation, Interleukin-6, murin pathogen free

Peripheral systemic inflammation has been suggested to increase cognitive decline in subjects without dementia or affected by neurodegenerative diseases such as Alzheimer's disease (AD) [1]. Only few studies have addressed the effects of systemic infections on the onset and progression of neurodegeneration in animals models of AD. Lipopolysaccharide (LPS)and viral infection worsens both tau and amyloid-β (Aβ)-related neurodegeneration [2–4].However, these studies did not address two important issues related to systemic inflammation: (1) the influence of the microbial environment in which mice are raised; (2) the effects of systemic inflammation in pre-symptomatic stages of the neurodegeneration development.

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We addressed these issues in the anti-Nerve Growth Factor (NGF) AD11 mouse, in which many features of the AD pathology are reproduced. AD11 anti-NGF mice develop, by a neurodegenerative phenotype characterized by cholinergic deficit, Aβ/APP immunoreactive dystrophic neurites in the hippocampus, tau hyperphosphorylation spreading from the cortex to the hippocampus and synaptic and memory deficits (reviewed in [5]). These phenotypic alterations are progressive, with a pre-symptomatic phase between birth and 2 months of age. Interestingly, a microarray gene expression analysis performed at a pre-symptomatic age, i.e. one month of age, showed a dramatic variation in genes related to inflammation at this stage [6]. All previous published work describing the neurodegenerative phenotype of AD11 mice was performed under conventional animal house conditions.

In this study, we verified whether an extrinsic factor such as the microbial environment in which AD11 mice are reared can influence the onset and

the progression of the AD-like neurodegeneration. A description of materials and methods is provided in Supplementary methods.

We first rederived AD11 mice by embryo transfer and kept them for generations under murine pathogen free (MPF) conditions. The genotype of rederived MPF mice was confirmed by PCR [7].

The comparison of the health reports from the two colonies (see Supplementary Table 1), revealed that CV-AD11 mice, but not MPF mice, have been exposed to three specific pathogens: murine*Norovirus, Helicobacter spp* and *Trichomonasmuris.*

We subsequently verified that MPF conditions were not affecting the expression of the recombinant anti-NGF antibody (Fig. 1A). The statistical analysis, performed using a was paired T-test showed no differences between the levels of transgenic anti-NGF antibodies in the blood serum of CV- and MPF-AD11 mice ($P = 0.06$).

Then, we verified the presence of the main features of neurodegeneration in 6 month-old AD11 mice both CV and MPF colonies. Based on previous work on CV-AD11 mice, we selected as read-outs for the neurodegeneration the cholinergic phenotype of basal forebrain neurons, the presence of Aβ-positive clusters of dystrophic neurites in the hippocampus and of phosphorylated tau in the cortex. At this age, neurodegeneration progressed at the stage that is not yet fully blown and is still fully reversible by pharmacological treatments [8, 9].

As, expected, the number of ChAT-positive neurons in CV-AD11 mice ($n = 9$) was decreased by 34% with respect to CV-WT mice (Fig. 1B,C,F; $P < 0.01$). A similar decrease was observed in MPF-AD11 mice with respect MPF-WT mice (Fig. 1D-F; $P < 0.01$).

Regarding APP and tau processing-related endpoints, an additional control group was included, the anti-NiP mice [10], created according to the same strategy used to produce the AD11 mice [7] but expressing an antibody against the biologically irrelevant antigen 4-Hydroxy-3-iodo-5-nitrophenylacetyl. Aβ-positive dystrophic neurites were observed in the hippocampus of CV-AD11 mice (Fig. 2A,D) while no Aβ/APP staining was detected in MPF-AD11 mice (Fig. 2B,D) or in CV-NiP control mice (Fig. 2C,D). Similarly, while intracellular accumulation of phosphorylated tau was found in the entorhinal cortex CV-AD11 mice(Fig. 2E,H), such alterations were not observed in age-matched MPF-AD11 mice (Fig. 2F,H) or in CV-NiP mice (Fig. 2G,H). Thus, the Aβ and phosphotau-related endpoints are only found

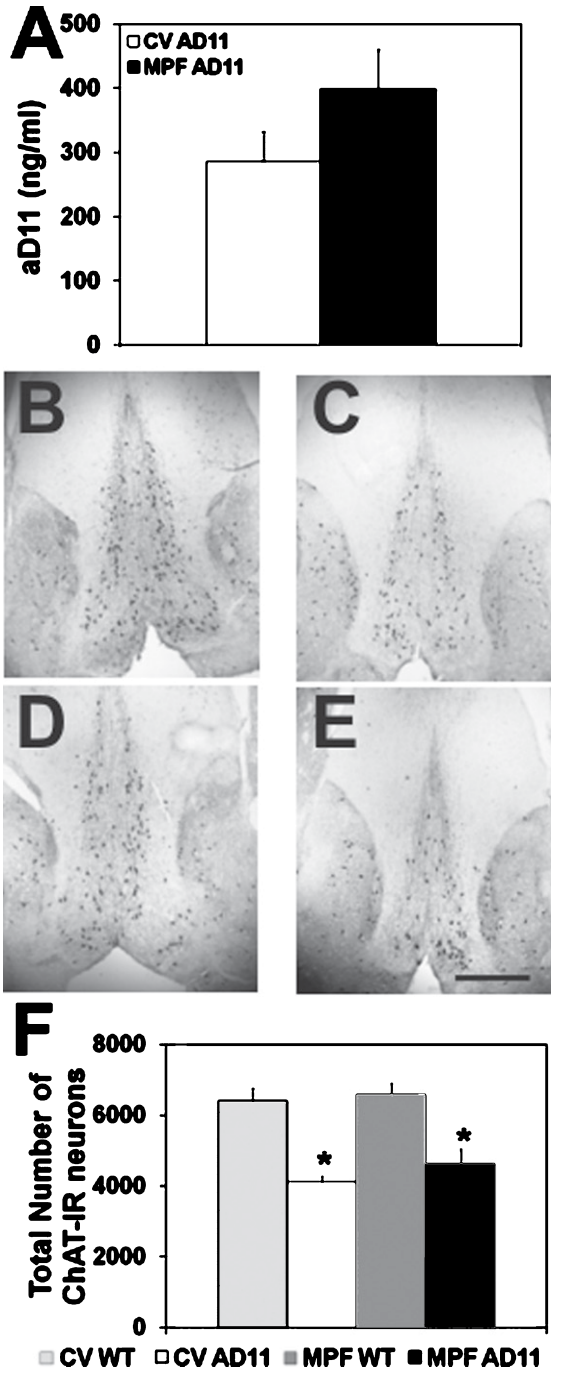

Fig. 1. Levels of recombinant anti-NGF antibodies and ChAT expression in CV and MPF AD11 mice. (A) The graph shows the mean value of serum anti-NGF antibodies for a group of CV-AD11 ($n = 18$) and MPF-AD11 mice ($n = 18$). No statistical difference was found ($P > 0.05$). (B,C) The number of cholinergic neurons is decreased in CV-AD11 mice (C) with respect to CV-WT mice (B). Similarly, MPF-AD11 mice show a decrease in ChAT-immunoreactive cells (E) with respect to MPF-WT mice (D). (F) Quantification of the cholinergic deficit in transgenic and WT mice. Bars represent mean ± s.e.m. Scale bar = 500 μm.

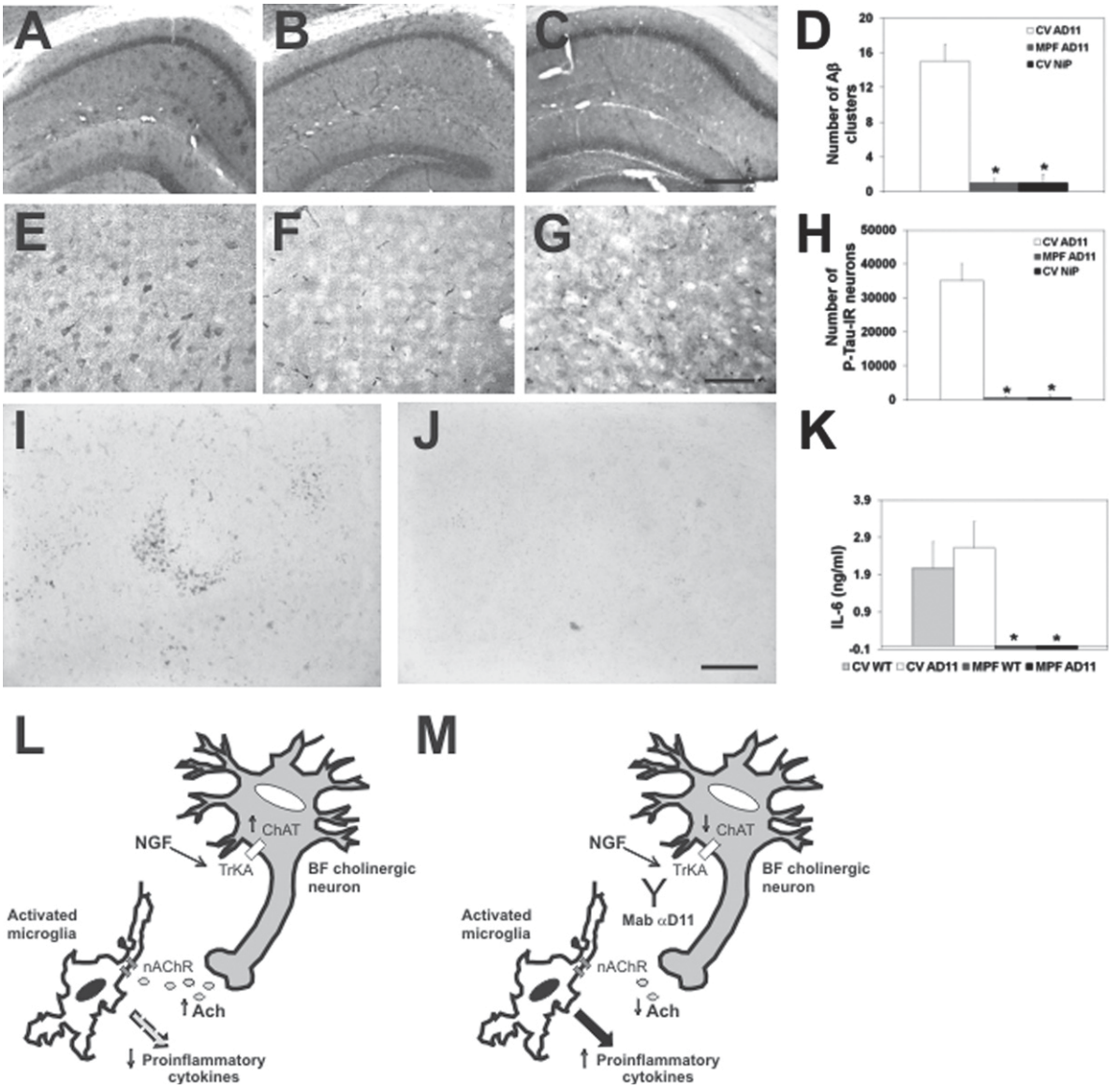

Fig. 2. Expression of neurodegenerative markersand and IL-6 serum levels in CV-and MPF-AD11 mice and in CV-NiP mice. CV-AD11 mice show clusters of Aβ/APP immunoreactive dystrophic neurites in the hippocampus (A), which can not be observed in MPF-AD11 mice (B) or in CV-NiP mice (C). (D) Quantification of Aβ clusters. In CV-AD11 mice, phosphorylated tau (mAb AT8) accumulates intracellularly (E). This accumulation is not observed in MPF AD11 mice (F) or in CV NiP mice (G). (H) Quantification of phosphotau immunoreactive neurons. CV AD11 mice show clusters of CD-45 immunoreactive microglial cells (I) which are completely absent in MPF mice (J).(K) Levels of Interleukin-6 in the four groups of mice. (L, M) Scheme illustrating a putative mechanism underlying anti-NGF deprivation. In (L), acetylcholine produced by basal forebrain cholinergic neurons binds to nicotine receptors (nAChR) expressed on microglia cells and decreases the release of proinflammatory cytokines. In (M), the transgenic antibody mAb αD11 neutralizes NGF activity and thus decreases the levels of choline acetyltransferase (ChAT). Consequently, lower amount of acetylcholine is released by cholinergic neurons, which in turn provokes an increased secretion of proinflammatory cytokines by microglia. Bars represent mean ± s.e.m. Scale bar in A-C = 200 μm; in E-G = 100 μm; inI-J = 50 μm.

when NGF activity is blocked and mice are kept in a conventional pathogen environment.

We evaluated markers of inflammation such as CD-45 in the hippocampus and IL-6 in the blood serum. A high number of CD45-immunoreactive microglial cells can be observed in CV-AD11 mice (Fig. 2I) and not in MPF-AD11 mice (Fig. 2J). IL-6 was undetectable in the blood serum of MPF-WT

and MPF-AD11 mice (Fig. 2K), while it reached the values of 2.82 ± 1 ng/ml in CV-WT mice and 4.08 ± 1.23 ng/ml in CV-AD11 mice (Fig. 2J).

Overall we conclude that, in presence of similar levels of recombinant anti-NGF antibody, MPF conditions can prevent amyloid deposition, tau hyperphosphorylation, while the cholinergic deficit is still present and that inflammatory and immune response were decreased in mice reared in MPF conditions, as assessed by the presence of microglia in the brain and by IL-6 levels in the blood serum.

Substantial evidence supports the concept that systemic inflammation during early postnatal life and adulthood contributes to exacerbate cognitive decline and disease progression in adult and elderly people [1, 11]. On the contrary, the link between early systemic inflammation and infections and chronic neurodegenerative diseases is still rather underexplored in animal models which would be useful to study the mechanisms involved. The effects of experimentally induced systemic inflammation (with LPS) have been studied in adult animal models of normal aging [12], acute neurodegeneration [13], ischemia [14], motor neuron disease [15], prion disease [16] and AD mouse models [2, 3]. In both APPswe and triple transgenic mice the injection of LPS was performed at ages in which the amyloid-related neurodegeneration was already ongoing, respectively at 11 and 4 months of age [2–4]. Thus, those studies addressed, in more detail, the effects of experimentally inflammation on the established chronic neurodegeneration and not on its onset.

In this study, we show that natural environmental exposure to bacteria, viruses and parasites during pregnancy and early postnatal stage of development can play an essential role in the development and severity of an AD-like neurodegeneration, triggered by an independent cause.

We found that MPF conditions not only prevented microgliosis in the brain, but also prevented the increase in Aβ/APP levels and tau hyperphosphorylation, while this condition did not affect the cholinergic deficit, which is a direct outcome of the anti-NGF antibodies in this model. Notably, Aβ/APP and hyperphosphorylated tau are not increased in control anti-NiP mice, confirming that NGF deprivation is fundamental for the onset of the neurodegeneration. These results are in line with studies in which rearing mice in specific pathogen free conditions prevents transthyretin-induced amyloidosis [17].

How can NGF deprivation and systemic inflammation co-operate on triggering neurodegeneration in AD11 mice? We have previously proposed a dual mechanism for neurodegeneration in AD11 mice, with a significant contribution by a facilitating immunotrophic and neuroinflammatory background [5]. The primary target of the anti-NGF antibodies are the cholinergic neurons of the basal forebrain, where NGF deprivation provokes atrophy [7, 18]. On the other hand, acetylcholine can inhibit the release of proinflammatory cytokines from microglia cells that express nicotinic receptors [19]. Thus a decrease of acetylcholine production by basal forebrain cholinergic neurons, as in the case of NGF deprivation, would provoke an increased secretion of proinflammatory cytokines by microglia (Fig. 2L,M). A second putative mechanism would be related to the fact that systemic infections can trigger an increased entry of IgGs in the brain and an augmented expression of Fcγ receptors on microglial cells [20], possibly activating the complement cascade. Overall, these mechanisms would increase the inflammatory state of the brain and worsen the neuroinflammation, contributing to the direct effects of neutralizing NGF [5]. In this context, the rescue of the neurodegeneration in AD11 mice, obtained after administration of NGF [8, 9]could be due not only to the neurotrophic activity of this neurotrophin but also to the interplay between NGF, which is also considered a neurokine [21], and other inflammatory molecules.

Is the mechanism revealed in AD11 mice relevant to human AD? AD patients, which are carriers of the APOe4 allele, are reported to have higher rates of CNS infection [22]. Several studies have reported a correlation between AD and pathogens [23], including *Helicobacter pylori* [24, 25], which belongs to the same species as one of the three pathogens that were found in CV AD11 mice and not in MPF AD11 mice. A selective exposure of formerly MPF-AD11 mice to each of the three microbial species identified in theCV-AD11 colony (*Norovirus, Helicobacter spp and Trichomonasmuris*) will allow to demonstrate whether any of these agents individually plays a direct role in creating a systemic environment facilitating the onset of neurodegeneration in the AD11 model.

Sickness behavior related to systemic inflammation is mediated by a disregulated expression of cytokines [26] and a specific correlation exists between an increase in plasma levels of IL-6 well before the appearance of clinical signs of dementia and AD neuropsychiatric symptoms [27, 28]. Interestingly, IL-6 plasma levels were higher in CV WT and AD11 mice, suggesting that this cytokine may

contribute, together with the anti-NGF antibody, to the development of the neurodegeneration.

In conclusion, the results described for the AD11 mice help elucidate the influence of systemic inflammation and innate immunity in the mechanism(s) contributing in the onset of AD neurodegeneration.

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SUPPLEMENTARY MATERIAL

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