Biochemical Changes in Disease

Edited by

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PREFACE

This book aims to understand and identify the main biochemical changes that occur in several diseases, namely pancreatic, cardiovascular, musculoskeletal, psychiatric, nutritional and infectious diseases. It is also focused on the recognition of possible biochemical markers of each disease, highlighting how they can be used in diagnosis.

Pancreatitis, cystic fibrosis and diabetes mellitus are the diseases of the pancreatic system, and many biochemical changes occur during the development and progress of these diseases. Cystic fibrosis is a common autosomal recessive disease and pancreas is mostly affected by cystic fibrosis. Diabetes mellitus occur due to a decrease in the amount of pancreatic beta cells as well as their dysfunction.

Cardiovascular diseases, mainly ischemic heart disease and stroke, with atherosclerosis as the key underlying factor, are one of the most common causes of death in both developing and developed countries worldwide, with an ever-increasing prevalence. Atherosclerosis results from low-grade chronic inflammation that arises from an interaction between immunological mechanisms and metabolic abnormalities within the vessel wall.

When considering musculoskeletal diseases, more than 150 possible diagnoses of conditions that affect the locomotor system should be considered. These conditions are characterised by pain, decreased physical abilities, big impacts in mental health and are a strong cause for the development of other chronic diseases such as obesity, since sedentarism and often incorrect food intake are associated. Musculoskeletal disorders are divided into three subgroups according to the affected organ or tissue, namely bone, joint and muscle diseases.

The American Psychiatric Association defines a mental disorder as a syndrome characterized by clinically significant disruption in an individual's cognition, behaviour, or emotion regulation, due to dysfunctions in psychological, biological or developmental processes concerning mental functioning. Nowadays common mental disorders are depression, anxiety and schizophrenia. Solid evidence on the aetiology and pathophysiology of these disorders are relevant for clinical psychiatry.

Nutritional diseases are any of the nutrient-related disorders and conditions that cause illness. These disorders occur when dietary intake does not contain the right amount of nutrients for healthy functioning of the body, or when nutrients from food cannot be properly absorbed. Nutritional diseases include a wide range of conditions, such as widespread undernutrition (malnutrition), overnutrition that leads to obesity, and the eating disorders such as anorexia nervosa, bulimia nervosa, binge eating disorder, and orthorexia nervosa and bigorexia.

Infectious diseases, caused by viruses, bacteria, and parasites, are a public health problem that has emerged in recent decades with new specificities. Infectious diseases can be acquired through direct contact with the infectious agent or through exposure to contaminated water or food, as well as through the respiratory or sexual route. Often, these diseases can also be transmitted from person to person. Different infectious diseases, namely COVID-19, AIDS, tuberculosis, *Helicobacter pylori*, *Tinea* and *Candida* infections, toxoplasmosis and malaria, can alter different biochemical parameters.

Chapter 1

Biochemical Changes in Pancreatic Diseases

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List of abbreviations

ADA: American Diabetes Association
ADIPOQ: Adiponectin
Akt: Protein kinase B
AP: Acute pancreatitis
ASL: Airway surface liquid
ATF6: Activating transcription factor 6
ATP: Adenosine triphosphate
BMI: Body mass index
CF: Cystic fibrosis
CFRD: Cystic fibrosis related diabetes
CFTR: Cystic fibrosis transmembrane regulator
CRP: C-reactive protein
DM: Diabetes mellitus
DNA: Deoxyribonucleic acid
EPO: Erythropoietin
ETS: Electron transport system
FoxO: Forkhead transcription factor

GAD: Glutamic acid decarboxylase
GADA: Glutamic acid decarboxylase autoantibodies
GCK: Glucokinase
GCKR: Glucokinase regulator
G-CSF: Granulocyte-colony stimulating factor
GDM: Gestational diabetes mellitus
GLUT-1: Glucose transporter 1
GSH: Glutathione
GSSG: Glutathione reductase
HbA1c: Glycated haemoglobin A1c
HGFs: Hematopoietic growth factors
IA-2: tyrosine phosphatase-related islet antigen *2*
IAA: İnsulin autoantibodies
ICAM-1: Inter-cellular adhesion molecule 1
ICM: Intestinal current measurement
IL: Interleukin
IRS1: İnsulin receptor substrate 1
IκB: Inhibitor of NF-κB
KCNQ1: Potassium voltage-gated channel subfamily Q member 1
M-CSF: Macrophage-colony stimulating factor
METSIM: The metabolic syndrome in men study
miRNA: microRNA
MTNR1B: Melatonin receptor 1B
NAD^+: Nicotinamide adenine dinucleotide
NEAPC: Neutrophil elastase antiprotease complex
NF-κB: Nuclear factor-kappa B
NMN: Nicotinamide mononucleotide
NPD: Nasi potential differential
OGTT: Oral glucose tolerance test
PCT: Procalcitonin
PI: Exocrine pancreatic insufficiency
PI3K: Phosphoinositide 3-kinase
PMN: Polymorphonuclear granulocyte
PPAR: Peroxisome proliferator-activated receptor
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RXR: Retinoid X receptor
SAA: Serum amyloid A
SHIP1: Src homology 2-containing inositol 5-phosphatase 1
SLPI: Secretory leukocyte peptidase inhibitor
SNP: Single nucleotide polymorphisms

TAP: Trypsinogen activation peptide
TCA: Tricarboxylic acid cycle
TCF7L2: Transcription factor 7 like 2
TNF- α: Tumour necrosis factor alpha
ZnT8: Zinc transporter-8

Introduction

Pancreatitis, cystic fibrosis (CF) and diabetes mellitus are the diseases of the pancreatic system, and many biochemical changes occur during the development and progress of these diseases. Over the past decades, especially in Western countries, an increase in both the incidence rate and hospital admissions have been reported for pancreatitis patients. Cystic fibrosis is a common autosomal recessive disease and pancreas is mostly affected by cystic fibrosis. According to the patient registry records of the Cystic Fibrosis Foundation, each year, almost 1000 new cases are being diagnosed with CF in the United States. Diabetes mellitus occur due to a decrease in the amount of pancreatic beta cells as well as their dysfunction. According to the American Diabetes Association (ADA), in 2018, approximately 10.5% of the population had diabetes mellitus and every year, more than 1.5 million new cases are being diagnosed in the United States.

Pancreas is an organ of the digestive system responsible for the production of insulin and other hormones as well as some important enzymes to breakdown foods. The pancreas plays a dual role: both an endocrine function and an exocrine function. It has an endocrine function as it secretes insulin and glucagon to regulate blood sugar. It has also an exocrine function as it secretes enzymes to help digestion. Among these enzymes, lipase digests fats, amylase digests carbohydrates and chymotrypsin and trypsin digest proteins.

In this section, the pancreatic system diseases are divided into three subgroups as pancreatitis, CF and diabetes mellitus. Biochemical changes that could be defined as the markers of the diseases are evaluated.

1.1. Pancreatic disorders

1.1.1. Pancreatitis

This is an inflammation of the pancreatic tissue. It may be caused by the pancreatic enzymes before they reach the duodenum. Acute pancreatitis

(AP) can be identified by a sudden and severe abdominal pain commonly caused by the blockage of the main pancreatic duct by gallstones. Fever and vomiting mostly accompany abdominal pain. It has also been reported that AP could be caused by taking too much alcohol. The morbidity and mortality rate is high in AP and it can turn into a chronic condition.

Biochemical markers in pancreatitis

C-reactive protein (CRP)

CRP is a biomarker for inflammatory diseases. After the onset of AP symptoms, CRP reaches its peak level within 72-96 hour. Mayer *et al.* (1984) reported that the severity of AP could be predicted by evaluating CRP levels. CRP has the advantages of having high prognostic value, being cheap and available in the routine clinical setting. However, liver disease can influence CRP levels and if the AP patient is obese and/or alcoholic, liver disease is mostly inevitable. Mikó *et al.* (2019) investigated the severity and mortality of AP by evaluating biochemical markers together with CRP and they found out that it has 71% sensibility and 87% specificity.

Procalcitonin (PCT)

PCT is the biologically inactive form of calcitonin and has been in use to detect the severity of AP during the last decade. It is superior to CRP as it can differ mild and severe AP within 24 hours. Since PCT is also the biomarker of both bacterial and fungal infections, sepsis and organ failure, it is nonspecific to AP. Khanna *et al.* (2013) reported that PCT could predict organ failure with 100% sensitivity and can predict severe AP with 86.4% sensitivity. Despite its high sensitivity, PCT is an expensive biomarker.

Interleukins (IL)

IL-1 is an important proinflammatory cytokine that can predict pancreatic necrosis within 48 to 72 hours with 88% sensitivity. Among the interleukins, IL-6 is the most promising biomarker for use in clinical routine. Soyalp *et al.* (2017) reported an elevation of IL-6 level in accordance with the severity of pancreatitis. Jiang *et al.* (2004) found 100% sensitivity and 89.7% specificity for the assessment of AP. Its major drawback is its high cost and the rapid drop of its serum concentration. IL-8 was found to be elevated significantly with the severity of AP. Rau *et al.* (1997) reported an association between IL-8 and pancreas necrosis. IL-8 increases rapidly (24 hours) after the onset of disease symptoms, but it is not recommended for AP diagnosis.

Other biochemical markers

Serum amyloid A (SAA) levels were found to elevate faster than CRP, but its sensitivity and specificity to AP were found to be slightly lower than CRP (69% and 67% versus 71% and 74%). SAA was also reported as being superior to CRP in terms of distinguishing mild and severe AP.

Trypsinogen activation peptide (TAP) is the amino terminal of trypsinogen which is known to be released from trypsinogen to peritoneum, serum and urine during AP. It induces the activation of proteases. A positive correlation was found between the level of pancreatic injury and activated proteases. TAP is superior to CRP as it has 100% sensitivity and 85% specificity to AP, but it cannot predict the progression of the disease as its concentration in the urine decreases rapidly.

Polymorphonuclear granulocyte (PMN) elastase is a serine protease found in the neutrophil granules. PMN elastase is an important AP biomarker as it is being released similarly to other factors such as proteolytic enzymes, reactive oxygen species, eicosanoids, cationic peptides and microbicidal products and reaches its peak concentration within 24 hours.

Tumour necrosis factor (TNF)-α is crucial for the detection of AP pathogenesis. High levels of TNF-α receptors were found in AP, being related to the severity of the disease.

Tissue factor is a transmembrane glycoprotein and *Hepcidin* is a circulating peptide hormone, both of which can assess AP severity better than CRP.

Soluble E-selectin and *soluble thrombomodulin* are endothelial markers which are found to be the predictive markers of mortality in AP.

Moreover, Milnerowicz *et al.* (2013) reported a strong correlation with AP severity and elevated *endothelin I* levels. Guo *et al.* (2012) verified a strong association between pancreatic necrosis and high *matrix metalloproteinase-9* levels. Zhou *et al.* (2019) reported *red blood distribution width* as a reliable marker for AP severity.

The elevation of *blood urea nitrogen* was also found to be correlated with mortality in AP patients. Elevated level of *haematocrit* was shown to be associated with pancreatic necrosis and AP severity. On the other hand, *hypoalbuminemia* and low serum *calcium* levels were reported as a predictor of severe AP. A link between pancreatic necrosis and elevated *creatinine* concentration was also reported by Muddana *et al.* (2009).

Low levels of *proteinuria* can be used as a marker to evaluate AP severity. Urine dipsticks are mostly being used for an easy and inexpensive detection of proteinuria. In severe AP patients, organ failure has been reported, being verified by measuring the level of *angiopoietin-2* which is known as an angiogenic growth factor that plays a role as a modulator of vascular permeability.

Intercellular adhesion molecule 1 (ICAM-1) can also be used as a marker for AP severity with the advantages of being a simple, rapid and reliable method.

1.1.2. Cystic fibrosis

Among the autosomal recessive diseases, CF is quite common and affects many organs, primarily the lungs and pancreas. This is due to a mutation in the cystic fibrosis transmembrane regulator (*CFTR*) gene. The *CFTR* gene is responsible for the formation of the CFTR protein. This protein has a channel structure in the membranes of cells that produce digestive, mucus, sweat, and salivary enzymes and plays a role in the passage of chloride ions into and out of these cells. Mutations in this gene can alter the production, structure or stability of the CFTR protein. Chloride cannot be transported to the cell surface as the CFTR protein becomes non-functional. With the deterioration of the structure of the mucus in the lungs, narrowing and obstructions are experienced in the airways. Similarly, it inhibits the secretion of digestive enzymes in the pancreas. As a result, eating disorders and growth retardation occur.

In CF, all organs in which the CFTR protein functions, are damaged. Lungs are among the most damaged organs with a frequency of 99%. After the lungs, the most affected organs are the reproductive organs and the pancreas. Obstructive azoospermia in the reproductive organs (97%), exocrine pancreatic insufficiency (87%), diabetes (32%) and pancreatitis (2%) may occur in CF patients. In addition, fatty liver (25-60%), cirrhosis (10%), cholecystolithiasis (15%) as well as meconium ileus (20%) and distal intestinal obstruction syndrome (6%) may occur.

In individuals with positive new-born screening or with CF symptoms or a family history of CF, the *sweat chloride test* is usually chosen for the diagnosis of this disease. This test measures the amount of chloride in sweat. A sweat chloride level of 30-59 mmol/L indicates the possibility of CF and if so, an additional test is needed. A chloride level of 60 mmol/L and above

is one of the diagnostic criteria for CF. CFTR genetic testing and CFTR function tests are also being used as additional tests for the diagnosis of CF.

Biochemical changes in lung, liver and pancreas

There is a loss of chloride and bicarbonate transport due to CFTR in the lung. As a result, airway surface fluid (ASL) dehydration and increased mucus concentration occur. The mucin content of goblet cells is elevated. Disruption of the mucus structure in the airways leads to airway obstruction and increased inflammation. Children with CF born with normal lung structure, but the occlusion of the bronchioles occurs when they become 4 months old. TNF-α, IL-1β, IL-6, and IL-8 were found to be elevated in the lung secretions of CF patients. Biliary obstruction occurs when CFTR function is impaired. This blockage causes various liver diseases. The most common of these diseases are hepatic steatosis, focal biliary cirrhosis and micro gallbladder. Studies show that the mechanical effects of flow in the apical membrane of cholangiocytes stimulate ATP release and affect chloride secretion, which further regulates bile secretion. Therefore, decreased bile flow resulting from CFTR dysfunction may exacerbate abnormalities in bile formation.

Pancreatic ductal cells secrete isotonic fluid in response to food intake. CFTR plays a crucial role in the production of this alkaline solution which helps to send the digestive proenzymes secreted from the pancreatic duct tree into the duodenum. It also neutralizes the acidic cumin entering the proximal part of the small intestine and the protons that are co-released during digestive enzyme secretion by the pancreatic acinar cells.

Damage to the pancreas is found in almost all patients with CF at the earliest stage. Pancreatic injury causes not only severe inflammation but also occlusion of ducts by mucoprotein plugs. Pancreatic cyst formation and fibrosis occur by the secretion of viscous and protein-rich fluid. The changes begin in utero and eventually lead to pancreatic insufficiency (PI), which manifests itself in 83% of all CF patients. The clinical symptoms of PI are abdominal pain, digestive upset and low BMI. Exocrine PI is a common gastrointestinal complication that affects people with cystic fibrosis. Pancreatic insufficiency results from a progressive fibrotic process that begins in the uterus. Pancreatic cells are damaged by the deposits of dehydrated pancreatic secretions and are replaced by fibrous scar tissue. The pancreas no longer functions effectively and produces reducing amounts of enzymes necessary for digestion. PI is the main cause of improper digestion of dietary macronutrients, including fat, protein and carbohydrate. If left

untreated, it will result in malabsorption symptoms such as malnutrition status, impaired growth and development, deficiency of fat-soluble vitamins and steatorrhea.

Cystic fibrosis related diabetes (CFRD)

CFRD has similar and different characteristics to type 1 and type 2 diabetes mellitus (DM). CFRD is characterized by insulin deficiency and insulin resistance as a result of the destruction of pancreatic islets. The prevalence of CFRD increases markedly with age and affects approximately 2% of children, 19% of adolescents, and 40% to 50% of adults. About 80% of individuals with severe mutations, have CFRD after the age of 40 and women have a higher prevalence. Features unique to CFRD include partial loss or dysfunction of pancreatic islets leading to the deficiency of insulin secretion. There is a chronic underlying inflammation that flares periodically during the infection which causes fluctuating levels of insulin resistance. Diabetes develops asymptomatically in CF patients and an annual screening with OGTT is recommended.

Biochemical markers in cystic fibrosis

The function of CFTR protein has to be monitored to identify CF. Biomarkers used for CF measure the function of CFTR protein in different tissues and organs. Today, *sweat chloride test*, *nasal potential difference (NPD)* measurements and *intestinal flow measurements (ICM)* are being performed to identify CF.

In the *sweat chloride test*, the sweat glands are stimulated with pilocarpine and the sweat is collected in a gauze or a collector. The amount of chloride in sweat is determined. A chloride level above 60 mmol/L indicates insufficient chlorine reabsorption and diagnosis of cystic fibrosis. *NPD* and *ICM* measure the voltage potential or electric current, respectively, arising from epithelial ion fluxes at the mucosal surface. The *NPD* measurement is thought to provide information on both sodium absorption and chloride excretion. When these two methods detect voltage potential or electric current difference, they help to estimate the ion transitions in cells, especially chloride.

Sweat chloride is a biomarker only suitable for systemic treatments in clinical trials. Significant changes in sweat chloride occurred after administration of the CFTR enhancer ivacaftor to CF patients with the G551D mutation. Subjects homozygous for the F508del mutation had minor changes after intervention with the CFTR corrector VX-809 and moderate

changes occurred during combination therapy with ivacaftor and VX-809. In patients with the nonsense mutation, ataluren improved NPD, but not sweat chloride.

Pulmonary biomarkers

As a result of finding the inflammation in the airway in CF and discovering the changes resulting from inflammation, various parameters have become candidate biomarkers for CF such as cytokines, neutrophil chemo attractants, proteases, antiproteases, adhesion molecules, antioxidants, nitric oxide metabolites, antimicrobial proteins, eicosanoids, mucins and components of signalling cascades.

In the lung tissues of CF patients, peroxisome *proliferator activating receptor* (PPAR) was found to be deficient when compared to the healthy controls. When activated, PPAR forms a heterodimer with the activated retinoid X receptor (RXR), which can modulate inflammation. PPAR typically exerts its attenuating effects by inhibiting NF-κB activity through upregulation of IκB or by competing with NF-κB for helicases. CF airway epithelial cell lines appear to have less PPARγ activity than non-CF airway epithelial cell lines. Therefore, decreased PPAR expression also contributes to the imbalance between IκB and NF-κB, possibly promoting increased inflammation in CF.

Metabolomic biomarkers

To understand epithelial dysfunction associated with CF mutations and to discover the biomarkers for therapeutic development, non-targeted metabolomic analysis was performed on primary human airway epithelial cell cultures of three individual CF patients and non-CF individuals. Statistical analysis revealed a number of reproducible and significant metabolic differences between CF and non-CF cells. Alongside changes consistent with known CF effects, such as decreased cellular regulation to oxidative stress and osmotic stress, new observations on cellular metabolism in disease have been established. In CF cells, the levels of various purine nucleotides were significantly reduced, which may function to regulate cellular responses through purinergic signalling. Moreover, CF cells exhibited reduced glucose metabolism in the glycolysis, pentose phosphate pathway and sorbitol pathway, which can further exacerbate oxidative stress and limit the epithelial cell response to environmental pressure. Taken together, these findings reveal novel metabolic abnormalities associated with the pathological process of CF and identify a

panel of potential biomarkers for therapeutic development using this model system.

It was observed that in CF cells, the levels of various purine nucleotides are significantly reduced. Moreover, CF cells exhibit reduced glucose metabolism in the glycolysis, pentose phosphate pathway and sorbitol pathway, which can further exacerbate oxidative stress. In a study, nucleotide, tryptophan, glutathione, glucose metabolisms and osmolytes were examined. One of the most important differences between CF and non-CF cells was found in nucleotide metabolism, during purine biosynthesis. In CF cells, the purine metabolites such as adenosine, inosine, hypoxanthine and guanosine were reported as significantly reduced. The cytidine metabolite of the pyrimidine metabolism also showed a decrease in CF cells. During tryptophan metabolism, 1-methylnicotinamide showed a dramatic 24-fold increase, while the level of its precursor, nicotinamide, was significantly reduced. A ~2-fold increase was observed in kynurenine and anthranilate levels in CF cells. Glutathione and its associated metabolites also demonstrated significant differences between CF and non-CF cells. Both oxidized glutathione (GSSG) and reduced glutathione (GSH) levels in CF cells were reduced to 30% of the amount present in non-CF cells. In addition, ophthalmate (Glu-2-aminobutyrate-gli), a metabolite involved in GSH synthesis, also showed similar decreases. S-lactoylglutathione, a metabolite derived from glutathione detoxification, was significantly lower in CF cells. Glucose has a central function in cellular metabolism to produce energy and biosynthetic precursors of nucleotides and fatty acids. The levels of glucose and various glycolytic intermediates, including glucose-6-phosphate, fructose-6-phosphate and lactate, were significantly reduced in CF cells. Ribulose-5-phosphate levels in the pentose phosphate pathway, malate levels in the tricarboxylic acid cycle, and sorbitol and fructose levels in the sorbitol pathway were found to be reduced. In general, these findings may show that glucose metabolism is suppressed in CF cells. The levels of the two main cellular osmolytes, sorbitol and glycerophosphorylcholine, were also found to be significantly reduced in CF cells compared to non-CF cells.

MicroRNA (miRNA) biomarkers

miRNAs are small, non-coding RNAs that participate in post-transcriptional gene expression. They are involved in many biological processes such as growth, development, differentiation, proliferation and cell death. In recent years, it has been shown that the expression of miRNAs in cystic fibrosis cell lines and tissues has changed, and studies have demonstrated that they

are one of the promising treatment approaches for the future. As a result of studies with different disease models, miRNAs that directly bind to CFTR mRNA or indirectly affect inflammation, fibrosis formation, CFTR protein folding, and many different mechanisms were shown to affect disease severity.

In CF, due to the formation of dehydrated mucus in the lung, chronic lung infection occurs following the reduction of CFTR channel activity. miRNAs indirectly affect disease severity in patients with CF by targeting genes involved in the immune response. In a study by Oglesby *et al.* (2010), it was found that the expression of miR-126 was decreased in primary bronchial cell cultures of patients with CF. In another study, it was shown that miR17 directly targets IL-8 and inflammation is triggered by reduced expression of miR17 in patients. miR-145, whose expression is increased in the nasal epithelial cell culture of CF patients, has been shown to trigger IL-8 release and inflammation by targeting SMAD3. In a study performed with primary bronchial cell cultures created from healthy and cystic fibrosis patients, it was determined that the expression level of miR-122, which directly targets the Activating Transcription Factor 6 (*ATF6*) gene, was increased in CF. ATF6 directs misfolded proteins to the degradation pathway. The CFTR protein, which cannot be destroyed, accumulates and creates endoplasmic reticulum stress.

Numerous miRNAs involved in inflammation pathways in cystic fibrosis have been identified. miR-155 is one of them. As a result of several studies in different cell models, it was found that miR-155 targets genes involved in the IL-8-mediated inflammation response. One of these genes, *SHIP1*, is a phosphatase enzyme involved in the PI3K/Akt signalling pathway. It was found that the *SHIP1* mRNA level decreased and the expression of miR-155 targeting *SHIP1* increased in the primary cell culture formed from nasal swabs taken from patients.

1.1.3. Diabetes mellitus

DM is a metabolic disease manifested as hyperglycaemia in the clinic under the influence of various genetic and environmental factors, resulting a decrease in pancreatic beta cells and their dysfunctions. All diabetics with hyperglycaemia are at risk for the same chronic complications, but the severity and progression of complications can vary. According to ADA criteria, DM can be classified under 3 headings:

Type I DM

Type 1 DM is defined as the persistent presence of two or more autoantibodies, together with clinical hyperglycaemia, having HbA1c levels between 5.7-6.4%, or more than 10%.

Type II DM

The first occurrence in the emergence of type 2 DM is insulin resistance. When insulin resistance occurs in the individual, the suppression mechanism of hepatic glucose production is impaired, and the use of insulin-mediated glucose in the muscles and liver decreases. Over time, this leads to loss of function due to excessive insulin secretion from the pancreatic β-cells and type 2 DM occurs.

Gestational DM (GDM)

GDM is a type of diabetes that can occur in women during pregnancy. It can be diagnosed in the second or third trimester of pregnancy. This situation is risky for the foetus and new-born. According to ADA, there are one-step and two-step strategies to diagnose GDM. One step strategy involves an oral glucose tolerance test (OGTT) with the ingestion of 75 grams of glucose. The OGTT needs to be performed in the morning after at least 8 hours of fasting. A value of fasting plasma glucose (FBG) above 92 mg/dL, together with a value of 1-h plasma glucose (1-h PG) more than 180 mg/dL and a two-hour plasma glucose (2-h PG) above 153mg/dL after OGTT, represents the presence of GDM.

According to the two-step strategy, a first non-fasting glucose load test (GLT), with ingestion of 50 grams of glucose, has to be applied. If the 1h-PG is between 130-140 mg/dL, then OGTT should be performed after fasting for at least 8 hours, with ingestion of 100 grams of glucose. In order to diagnose GDM, at least two of the four following PG levels must be present: FBG above 95 mg/dL; 1-h PG above 180 mg/dL; 2-h PG above 155 mg/dL; and 3-h PG above 140 mg/dL.

Biochemical markers in diabetes mellitus

Glycated haemoglobin A1c (HbA1c)

HbA1c is the most important marker of blood glucose levels in patients with diabetes mellitus. This marker occurs as a result of changes in the haemoglobin molecule. It is widely used for routine glycaemic monitoring

in patients with type 1 and type 2 diabetes. HbA1c (a sub-fraction of glycosylated haemoglobin), produced at a rate dependent on substrate (glucose) concentration, is continuously formed *in vivo* by glucose forming a keto amine at the N-terminal of the haemoglobin beta chain. Glucose enters erythrocytes at a rate proportional to extracellular concentration via constitutively active GLUT1 channels, so the intracellular and extracellular glucose environments are nearly equivalent. The unique microvascular complications of diabetes (nephropathy, neuropathy, and retinopathy) occur in tissues that also express the GLUT1 channel and are likely caused by intracellular glucose toxicity. Since the half-life of erythrocytes is 120 days, HbA1c measurement estimates the average glycaemic index of the last 3 months. HbA1c measurement has several advantages over fasting glucose measurement and OGTT. These advantages can be counted as not having to fast, not requiring preanalytical stability and not being affected by environmental and personal factors. During pregnancy, HbA1c shows biphasic changes that decrease between the first and second trimesters and increase in the third trimester.

Fructosamine

Fructosamine is an alternative diagnostic criterion used as a glycaemic marker. Fructosamine is a glycoprotein that is formed by a covalent linkage between a sugar (such as glucose or fructose) and total serum proteins, particularly albumin, thus forming keto amines. It reflects the average blood glucose level over the past 14 weeks. This method is cost-effective and easy. It is also a good indicator for microvascular complications. It is often used to evaluate glycaemic control to monitor the effectiveness of treatment, often when a patient changes medication or insulin. Recently, fructosamine has been associated with all-cause and cardiovascular disease mortality and morbidity in haemodialysis patients. Gounden *et al.* (2021) reported a reference range for fructosamine in non-diabetic individuals as 200 to 285 µmol/L. Fructosamine levels increase in conditions of high glucose concentrations, such as diabetes. In a study conducted by Kalia *et al*. (2004), it was found that the fructose values of patients with a long-term history of diabetes and chronic hyperglycaemia were higher than the fructose values of healthy people without diabetes.

Glycated albumin (GA)

GA represents glucose levels for a 2-weeks period, similar to fructosamine, and increases in conditions of high glucose concentration such as diabetes. In some special cases like renal failure and haemolytic anaemia, it may be

a better indicator than HbA1c. Under conditions of hyperglycaemia, albumin glycation can occur as a result of spontaneous, non-enzymatic Maillard reaction.

Albumin is known to be more sensitive to glycation than haemoglobin. Pathological conditions such as nephrotic syndrome, hepatic cirrhosis and thyroid diseases can affect blood levels of glycated albumin. During diabetes treatment, the level of glycated albumin decreases more rapidly than HbA1c. Therefore, it is thought that glycated albumin may be important in providing therapeutic observation. Since it is not affected by anaemia and related treatments, it has become a prominent biomarker in glycaemic control in dialysis patients.

Iron deficiency is common in the first trimester of pregnancy. In this period of gestational diabetes patients, measurement of glycated albumin instead of HbA1c may provide an advantage in blood glucose monitoring since it is not affected by iron deficiency treatment.

Insulin and C-peptide

Insulin and *C-peptide* serum concentrations elevate in case of insulin resistance that occurs before the development of Type 2 DM. The deterioration of beta cell function over time causes a reduction in insulin and C-peptide serum concentrations. After proinsulin formation in the endoplasmic reticulum, proinsulin is packaged in the form of granules and, by limited proteolysis, cleaved into insulin and C-peptide. Upon stimulation (e.g., by glucose), both peptides rapidly secrete into the circulation at equimolar rates. Because insulin is rapidly degraded by liver, peripheral blood insulin concentrations are significantly reduced when compared to C-peptide levels. Under *in vivo* conditions, C-peptide has a longer plasma half-life and is less affected by haemolysis than insulin, and standardization studies are already well advanced. Determination of C-peptide concentration is also important for the distinction between type 1 and type 2 diabetes and for classification of diabetes subtypes.

Wnt proteins

Wnt proteins determine embryonic cell proliferation, cell differentiation and multiple cellular functions for the survival of cells including neurons, cardiomyocytes, endothelial cells, red blood cells, tumours, and adipose tissue. Recent studies have found that an abnormality in the Wnt pathway increases the risk of type 2 DM and a strong association with obesity was reported. In patients with DM, an elevated expression of Wnt-5b in adipose

tissue, liver and pancreas was found. It was also noticed that the expression of Wnt-3a and Wnt-7a was increased in hyperglycaemic mice fed with high amounts of fat.

Intact Wnts increase glucose tolerance and insulin sensitivity. It has also been observed that intact Wnts can protect glomerular mesangial cells from high glucose-induced apoptosis. Improved glucose homeostasis and reduced hyperinsulinemia were observed in mice overexpressing Wnt-10 on a high-fat diet. Wnt-1, controlled by erythropoietin (EPO), protects cells from high glucose exposure.

Wnt improves cellular protection in patients with DM through regulation of protein kinase B (Akt). Akt activation supports cell survival in cases of cell proliferation, progenitor cell development, permeability of the blood-brain barrier, and inflammation. In addition, Akt can modulate microglial cell activation, regulate transcription factors, inhibit cytochrome c release, and block caspase activity. Thus, Wnt can prevent the occurrence of oxidative stress through Akt in diabetes. Silencing *Akt* gene expression and inhibition of the phosphatidylinositol 3-kinase (PI3-K) pathway can prevent Wnt from blocking apoptotic damage.

Nicotinamide/nicotinic acid

Nicotinamide/nicotinic acid is the water-soluble form of vitamin B3 and is rapidly absorbed by the gastrointestinal epithelium. Nicotinic acid is converted into nicotinamide in the liver or by NAD^+ hydrolysis. It is the precursor of adenine dinucleotide (NAD^+). Nicotinamide can be converted into nicotinamide mononucleotide (NMN) by nicotinamide adenylyl transferase. NAD^+ synthase or nicotinamide riboside kinase catalyses the conversion of NMN to NAD^+. NAD^+ takes part in energy metabolism in the tricarboxylic acid (TCA) cycle. It plays an active role in the production of ATP, DNA synthesis and repair in the electron transport system (ETS).

In case of exposure to reactive oxygen species (ROS) products, nicotinamide/nicotinic acid can increase endothelial cell viability. Just before DNA damage occurs in the cell, nicotinamide intervenes. Thus, it can prevent apoptotic damage. This suggests that apoptotic damage may be reversible. Nicotinamide/nicotinic acid provides protection against free radical formation in neuronal cells.

Since nicotinamide plays an important role in cellular energy management, it is thought to be important during diabetic complications. It maintains fasting blood sugar in streptozotocin induced diabetic mice. In a clinical

study, it has been shown that oral nicotinamide administration (1200 mg/m^2/day) inhibits type 1 DM islet cell antibody formation and protects pancreatic beta cell function.

Some studies have shown that long-term nicotinamide exposure reduces pancreatic beta cell function. They stated that it slows down cell growth and may promote DM. It can also cause the progression of diseases such as Parkinson's by inhibiting cytochrome P450 and hepatic metabolism.

A new transcription factor that can control some of the beneficial effects of nicotinamide in DM is forkhead (FoxOs). They bind to DNA and activate or inhibit target genes. FoxO proteins play a cytoprotective role in DM and cell metabolism. FoxO-1 improves glucose tolerance and insulin sensitivity by its expression in adipose tissue during high-fat diet in mice. FoxO3a controls apoptotic damage via caspase. Thus, it contributes to cell viability. Some studies have shown that FoxO1, FoxO3a and FoxO4 reduce post-translational phosphorylation and can initiate cellular apoptosis. Like the Sirt-1 activator resveratrol, increased transcriptional activity of the *FoxO1* gene may reduce insulin-mediated glucose uptake and insulin resistance may occur. Overexpression of *FoxO1* in skeletal muscles reduces muscle mass and attenuates glycaemic control in mice. Nicotinamide inhibits the activity of FoxO proteins. It blocks apoptotic cell damage by FoxO3a phosphorylation and inhibits caspase 3 activity.

Nicotinamide prevents oxidant-induced apoptotic damage in a certain serum concentration range. Nicotinamide protects cells against oxidative stress at a concentration of 5-25 mmol/L.

Erythropoietin (EPO)

EPO, a growth factor and cytokine, is approved by the Food and Drug Administration for the treatment of anaemia. But clinical studies have shown that apart from anaemia, it can also be a treatment agent in conditions such as Alzheimer, depression, cardiovascular diseases, spinal cord injury, ocular and gastrointestinal disorders.

The main organs where EPO is produced are the liver, kidney, brain, and uterus. The amount of EPO is relatively lower in patients with DM, regardless of anaemia. EPO secretion increases in gestational diabetes. EPO is thought to be elevated when the body defends itself against DM. In animal experiments, EPO can reduce apoptotic pathways in the developing brain under the conditions of hyperoxia. In addition, EPO can prevent the toxic effects of agents used in cognitive control such as haloperidol.

In diabetic and non-diabetic patients with severe congestive heart failure, EPO reduces fatigue, significantly reduces hospital stay, and increases left ventricular ejection fraction. Also, EPO can reverse complications of anaemia that can occur during DM.

EPO can drive the modulation of FoxO proteins and Wnt signalling. In this way, it can have protective effects both in the haematological and vascular system. Cell culture studies have shown that Wnt1 protein is sufficient for cellular protection at high glucose exposure. EPO is involved in the maintenance of mitochondrial membrane potential. In case of disruption of membrane mitochondrial potential, apoptotic damage may occur in the cell. It has been shown that EPO can provide protection in cellular apoptosis and prevent mitochondrial depolarization.

When metabolite biomarkers were examined, a relationship between type 2 DM and amino acid metabolism was determined. Some amino acids, such as valine, leucine, isoleucine and tryptophan, were found to have higher serum concentrations in T2DM patients. In a meta-analysis study by Long *et al.* (2020), some serum metabolites of T2DM patients were examined. The levels of valine, leucine, isoleucine, proline, tyrosine, lysine and glutamate were found to be lower and glycine higher than normal levels. In the Male Metabolic Syndrome (METSIM) cohort study, which included only men and had 4.5 years of follow-up, various metabolites were measured and their relationship with type 2 DM was examined. Among the metabolites, mannose was shown to have the strongest association with type 2 DM. Mannose was found to be inversely proportional to insulin sensitivity and insulin secretion, but its mechanism was not elucidated. Mannose is required for glycoprotein synthesis. In addition, proinsulin, lipids, glycerol, non-cholesterol sterols, isoleucine and alanine, acetoacetate and some inflammatory markers were shown to be associated with type 2 DM. The effect of lifestyle and environmental factors on pancreatic beta cell functions is lower than in insulin sensitivity. Therefore, the measurement of pancreatic beta cell functions is the main focus in biomarker studies for Type 2 DM.

Autoantibodies

Autoantibodies are being used to distinguish between autoimmune (type 1) and non-autoimmune (mainly type 2) diabetes, and to predict the requirement for insulin therapy. Clinically, adult-onset diabetes, that is mostly seen over the age of 30, does not show any signs of ketoacidosis and weight loss. It is a slow-growing form of autoimmune type 1 DM, also called GADA

(Glutamic Acid Decarboxylase Autoantibodies). It is often misdiagnosed as type 2 DM and treatment is performed accordingly. Therefore, it is important to conduct autoantibody tests in diabetic adults to determine the correct diagnosis and treatment.

Classical biomarkers that come to the fore in type 1 DM are serum autoantibodies against beta cell antigens, including *insulin (IAA), glutamic acid decarboxylase (GAD), tyrosine phosphatase-like protein (IA-2)* and *zinc transporter 8 (ZnT8).* The formation of beta cell autoantibodies is generally observed 6 months after birth. The first autoantibodies formed are usually IAA at 9-24 months of age and GADA at 36 months. While 70% of individuals with diabetes have three or four autoantibodies, only 10% have a single autoantibody. Diabetes diagnosed in childhood mostly contains IAA autoantibodies. Since IA-2 autoantibodies are seen in almost all type 1 DM patients, it is considered as an important biomarker. Similarly, ZnT8 autoantibodies are commonly seen in type 1 DM patients, thus facilitating the predictability of the disease. High levels of IAA and IA-2 increase the development of type 1 DM according to the results of the TEDDY study.

Single nucleotide polymorphism (SNP)

Many studies reported a relationship between *single nucleotide polymorphism (SNP)* and GDM. The transcription factor 7-like 2 (*TCF7L2*) gene encodes a transcription factor involved in Wnt signalling, an important signalling pathway in regulating glucose homeostasis. Most studies investigating four different SNPs in this gene (rs7903146, rs4506565, rs7901695 and rs12255372) found an association between the T allele (rs7903146) and GDM.

Adiponectin (ADIPOQ), an adipokine that regulates glucose and lipid metabolism, has been associated with GDM. G allele of rs266729 and rs2241766 SNPs formed in this *ADIPOQ* gene was associated with GDM. The melatonin receptor 1B (*MTNR1B*) gene encodes one of the melatonin receptors involved in regulating insulin signalling and glucose metabolism. Three studies investigating Rs1387153, one of the SNPs in this gene, reported an association between the T allele and GDM. Studies with glucokinase (*GCK*) and glucokinase regulator (*GCKR*), insulin receptor substrate 1 (*IRS1*), potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*) genes SNPs demonstrated that some variants are associated with GDM.

Conclusion

In this chapter, most commonly seen pancreatic disorders (pancreatitis, CF and diabetes mellitus) were briefly described. The biochemical changes reported in the outcome of several clinical studies were evaluated. These biochemical changes reflect the differences in cellular pathways and help to understand the metabolism dependent mechanisms of the diseases. In pancreatitis, although nonspecific to AP, PCT is more sensitive to AP than CRP. IL-6 is reported as the most promising biomarker for clinical usage as it has high specificity and sensitivity to AP. Biomarkers (pulmonary, metabolomic and miRNA) of CF measure CFTR gene in diverse tissues and organs. In diabetes mellitus, together with glucose, HbA1c is the most crucial diagnostic marker. For the classification of diabetes mellitus, biomarkers such as insulin, C-peptide and autoantibodies are mostly being used. Standardization of each biomarker for the related condition is important to be able to list in the clinical practice guidelines to ease the diagnosis, classification and monitoring of the diseases. We believe that the information being provided in this chapter will enlighten future studies.

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Chapter 2

Biochemical Changes in Cardiovascular Diseases

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List of abbreviations

AA: Arachidonic acid
ADM: Adrenomedullin
ADMA: Asymmetric dimethylarginine
ADP: Adenosine diphosphate
AMPK: Adenosine monophosphate-activated protein kinase
Apo: Apolipoprotein
ATG6: Autophagy protein 6
Bcl: Beclin
CETP: Cholesteryl ester transfer protein
CRP: C-reactive protein
CVD: Cardiovascular diseases
COX-1: Cyclooxygenase-1
DAG: Diacylglycerol
DHA: Docosahexaenoic acid
DM: Diabetes mellitus
DNA: Deoxyribonucleic acid
ECM: Extracellular matrix
eNOS: Endothelial nitric oxide synthase

EPA: Eicosapentaenoic acid
GDF15: Growth/differentiation factor 15
GPVI: Glycoprotein VI
GPx: Glutathione peroxidase
HDL: High-density lipoproteins
HDL-c: High-density lipoproteins cholesterol
HMG-CoA: 3-Hydroxy-3-methyl-glutaryl-coenzyme A
hs-CRP: High sensitivity assays to quantify the low C-reactive protein
IBD: Inflammatory bowel disease
ICAM-1: Intercellular adhesion molecule 1
Ig: Immunoglobulin
IL: Interleukin
IP3: 1,4,5-Trisphosphate
LDL: Low-density lipoproteins
LDL-c: Low-density lipoproteins cholesterol
LDL-r: Low-density lipoproteins receptors
LFA-1: Lymphocyte function-associated antigen 1
LOX-1: Lectin-like oxidized low-density lipoprotein receptor 1
Lp(a): Lipoprotein(a)
Lp-PLA2: Lipoprotein-associated phospholipase A2
MCP-1: Monocyte chemoattractant protein 1
miRNA or miR: Micro ribonucleic acid
MMP-9: Matrix metalloproteinase 9
MPO: Myeloperoxidase
MR-proADM: Midregional pro-adrenomedullin
mTOR: Mechanistic target of rapamycin
NADPH: Nicotinamide adenine dinucleotide phosphate
NOX: Nicotinamide adenine dinucleotide phosphate oxidases
OPN: Osteopontin
OPG: Osteoprotegerin
oxLDL: Oxidized low-density lipoproteins
P2Y: Peptide 2Y
P53: Tumour protein 53
P62: Tumour protein 62
PA: Plasminogen activator
PAI: Plasminogen activator inhibitors
PAPP-A: Pregnancy-associated plasma protein-A
PCSK-9: Proprotein convertase subtilisin/kexin type 9
PDGF: Platelet-derived growth factor
PECAM-1: Platelet endothelial cell adhesion molecule 1
PIGF: Placental growth factor

PN-1: Protease nexin 1
PPAR-α: Peroxisome proliferator-activated receptor-α
PTP: Protein tyrosine-phosphatases
RA: Rheumatoid arthritis
RNA: Ribonucleic acid
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SAA: Serum amyloid-A protein
sCD40L: Soluble CD40 ligand
SLE: Systemic lupus erythematosus
sPLA2: Secretory phospholipase A2
SOD: Superoxide dismutase
TXA2: Thromboxane A2
TG: Triglycerides
TNF-α: Tumour necrosis factor-α
tPA: Tissue plasminogen activator
TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand
uPA: Urokinase plasminogen activator
VCAM-1: Vascular adhesion molecule 1
VLA-4: Very late antigen 4
VSMC: Vascular smooth muscle cells
XO: Xanthine oxidase

Introduction

Cardiovascular diseases (CVD), mainly ischemic heart disease and stroke, with atherosclerosis as the key underlying factor, are one of the most common causes of death in both developing and developed countries worldwide, with an ever-increasing prevalence. Total CVD cases nearly doubled from 271 million in 1990 to 523 million in 2019, and the number of CVD deaths increased from 12.1 million in 1990 to 18.6 million in 2019. Nevertheless, CVD mortality rates have continued to decline worldwide. In the last three decades, more than half of the reduction in CVD mortality has been attributed to changes in risk factor levels in the population, primarily the reduction in cholesterol and blood pressure levels and smoking. Other risk factors, as unhealthy diet, physical inactivity, harmful use of alcohol, obesity and type 2 diabetes mellitus (DM), together with non-modifiable factors, as sex and age, partly offset this favourable trend.

Atherosclerosis results from low-grade chronic inflammation that arises from an interaction between immunological mechanisms and metabolic

abnormalities within the vessel wall. In addition to inflammation, risk factors for this condition include high cholesterol and low-density lipoproteins (LDL), low level of high-density lipoproteins (HDL) in the blood, hypertension, tobacco smoke, diabetes mellitus, obesity, inactive lifestyle, unhealthy diet, body mass index, waist circumference, older age, family history of early earth disease, high blood levels of triglycerides (TG), inflammation, sleep apnoea, stress, alcohol consumption.

This chapter concentrates on the pathogenesis and biochemical changes of atherosclerosis, that is the major cause of CVD, and it is driven by oxidative stress and enhanced inflammation in the artery wall.

2.1. Pathogenesis of atherosclerosis

Blood vessel thickening due to the formation of plaques in the subendothelial intimal space is the main feature of atherosclerosis. Cholesterol, TG and lipoproteins are directly related to the pathogenesis of this disease. Atherosclerosis begins with the accumulation of LDL, which are sequestered in the subendothelial space by adhesion to extracellular matrix proteins rich in proteoglycans. This vessel thickening is more frequent in coronary artery, carotid artery, abdominal aorta, descending aorta, and iliac artery.

When LDL reaches the sub-intimate space, it can be aggregated and/or oxidized, becoming strong chemo attractants. The aggregation of LDL gives rise to complexes, which can undergo pinocytosis or phagocytosis by macrophages, that become foam cells. Macrophages are originated from circulating monocytes, that adhere to the endothelial cells that express adhesion molecules, such as vascular adhesion molecule 1 (VCAM-1), monocyte chemoattractant protein 1 (MCP-1) and P and E-selectins and migrate via diapedesis in the subendothelial space. Monocytes differentiate into macrophages and engulf oxidized LDL, becoming foam cells and contributing to plaque/atheroma development by secreting multiple mediators of the inflammatory process.

The increased inflammation triggers the migration of vascular smooth muscle cells (VSMC) from the tunica media into the subendothelial space where they abnormally proliferate and secrete extracellular matrix (ECM) proteins, contributing to atheroma growth. VSMC present in the intimal layer form a fibrous cap that contains the plaque. The atheroma plaque is mainly composed of a mixture of macrophages, lymphocytes, VSMC, cholesterol, necrotic debris, and foam cells. In the advanced stages, there is

intra-plaque neovascularization and haemorrhages. Platelet aggregation and clotting activation have a role in the development of thrombotic complications by adhering to the exposed sub endothelium at the site of plaque rupture and erosion. The rupture of the fibrous cap leads to thrombus formation causing blockage of the blood flow, or embolism in areas of the vascular bed far from the atherosclerotic area.

Thus, according to Bergheanu et al. (2017), the vascular modifications observable in atherosclerosis are progressively:

1- Intimal thickening, with deposition of VSMC and ECM proteins;
2- Fatty streak, where macrophage foam cells develop, mixed with VSMC;
3- Pathologic intimal thickening: VSMC and ECM proteins aggregate near the lumen, over an acellular area rich in hyaluronan and proteoglycans with lipid infiltrates;
4- Fibroatheromas, with an acellular necrotic core covered by a thick fibrous cap of VSMC in proteoglycan-collagen matrix;
5- Vulnerable plaque, with a thin type I collagen fibrous cap;
6- Ruptured plaque: the rupture of the fibrous cap leads to the presence of luminal thrombus and the increased macrophage infiltration.

The initial phase of atherosclerosis, which can last decades, is asymptomatic, as the plaque forms in the vessel wall of multiple arterial beds in proximity to bifurcations. The progressive narrowing of the arterial lumen is initially counterbalanced by vasodilation of arteries and collateral vascularization. The reduction of blood flow caused by a stenosis, which overcomes the reserve dilation capacities of the arteries, leads to the clinical features of atherosclerosis, which are angina pectoris, intermittent claudication or a transient ischemic attack. If an acute thrombotic obstruction, triggered by plaque disruption or, more frequently erosion, took place, an acute coronary syndrome or a stroke can occur.

2.2. Oxidative stress in cardiovascular diseases

The heart needs adequate oxygen supply to maintain its contractile function. At the cellular level, oxygen undergoes a reduction to superoxide anion (O_2^-) through the action of different types of oxidases (*e.g.,* uncoupled endothelial nitric oxide synthase (eNOS), mitochondria and xanthine oxidase (XO), NADPH oxidases (NOX)). Reactive oxygen species (ROS) are subcellular messengers in signal transduction pathways with beneficial and harmful functions. Under physiological conditions, ROS, as reactive nitrogen

species (RNS), play important roles in different signalling pathways, through the oxidation of specific targets. However, after increasing the activation of ROS/RNS-producing enzymes and/or in the face of a deficiency in endogenous antioxidant capacity, oxidative stress may occur. This can lead to reversible changes that can transiently alter the activity of the protein involved in the physiological adaptations, or irreversible oxidations, which originate pathophysiological processes. ROS are by-products of mitochondrial respiration or metabolism, being produced by specific enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, peroxiredoxins and myeloperoxidase. In the heart and skeletal muscle, ROS are essentially originated by NOX, eNOS and XO, producing superoxide anion and/or hydrogen peroxide (H_2O_2). O_2^- reacts with nitric oxide (NO) and forms peroxynitrite ($ONOO^-$), that is a very RNS. H_2O_2 and O_2^- are the predominant redox signalling agents originated under the control of growth factors and cytokines by more than 40 enzymes, prominently including NOX and the mitochondrial electron transport chain. However, several other reactive species are involved in redox signalling, such as nitric oxide, hydrogen sulphide and oxidized lipids. ROS/RNS, that are produced in cardiomyocytes in response to specific stimuli (acute, transient or sustained), originate lipid peroxidation, interact with DNA repair enzymes and transcription factors or cause DNA damage, lead to the oxidation/nitration of key proteins involved in contractility, calcium manipulation, metabolism, antioxidant defence mechanisms, among others. ROS/RNS also stimulate the inflammatory process, signs of stress inducing cardiac hypertrophy, fibrosis or cell death via apoptosis/necrosis and deregulate autophagy. ROS involved in signalling cardiac redox may have several origins, but NADPH oxidases, as dedicated sources of signalling of reactive oxygen species, seem to be of great importance. In fact, NADPH oxidases are a family of enzymes whose primary function is to produce ROS, mediating adaptive and maladaptive changes in the heart. The activity of NADPH oxidases is increased in the diabetic heart, characterized by an increase in oxidative stress.

There are 7 different isoforms of NOX (NOX1-NOX5 and DUOX1 and 2), being NOX1, 2, 4 and 5 identified and characterized in the cardiovascular system. Since cardiomyocytes contain a high concentration of mitochondria, which provide the main source of endogenous ROS, these organelles suffer oxidative damage, which often leads to the death of apoptotic cells and initiates cardiac pathology.

NOX4 has its highest levels of expression in proximal tubular cells of the kidneys, but is also expressed in other types of cells, including cardiomyocytes.

In the cardiovascular system, an increase in NOX4 expression can be triggered by various situations, such as pressure overload, hypoxia, and inflammation, which significantly affects cell function. This isoform essentially produces H_2O_2. Although NOX4 may play a protective role in cases of cardiac hypertrophy, fibrosis, contractile function, harmful effects are observed on the overloaded heart due to increased ROS production and consequent mitochondrial damage.

2.3. Haemostatic process activation

The haemostatic process can be divided in three steps: primary haemostasis, in which an interaction between platelets and the damaged endothelium occurs; secondary haemostasis, in which the activation of the clotting system happens, consists of activation of the protease cascade resulting in the production of fibrin clots; and the last step is fibrinolysis, a counterbalance system that degrades fibrin and dissolves the clot.

Primary haemostasis involves the adhesion, activation, and recruitment of platelets. The activated platelets release proinflammatory and pro aggregating factors from their granules, as adenosine diphosphate (ADP) and thromboxane A2 (TXA2), inducing the production of secondary messengers, such as diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) which, in turn, lead to an increased concentration of cytosolic calcium. Studies showed that ROS are able to mimic the roles of ADP and IP3 by influencing primary haemostasis through platelet activation. Platelets could be a target of ROS but also a source of free radicals. Indeed, an imbalance between ROS production and the antioxidant system dysregulates and amplifies platelet activation due to isoprostane formation, the modulation of platelet receptors, and the oxidation of LDLs. Platelet activation, in turn, leads to further ROS production through NOX activation, triggering mitochondrial dysfunction (Fig. 2-1).

Secondary haemostasis can also be impaired by ROS, especially those generated by NOX, promoting clotting activation, as they are able to upregulate the tissue factor, causing prothrombotic effects within blood vessels. ROS overproduction provokes the reaction of NO with O_2^-, generating $ONOO^-$, which, in turn, leads to serine-protease and fibrinogen nitration resulting in a pro-coagulant environment. Besides the increase and dysregulation of coagulation cascade factors, thrombosis occurs following a decrease in anticoagulant factors, such as protein S. Fibrinolysis consists of plasminogen activator (PA) stimulation to produce plasmin, which is able to promote the lysis of fibrin, accelerating clotting degradation. However, a

relative increase in the plasmin concentration is avoided through the actions of plasminogen activator inhibitors (PAI-1 and PAI-2). ROS are able to upregulate PAI-1, which is also implicated in the development of the atherothrombotic process. There is experimental evidence showing that PAI-1 expression is strongly increased during the inflammatory process and during atherothrombogenesis, and studies have shown that the oxidation of LDL, with consequent formation of oxLDL, induces the overproduction and release of PAI-1.

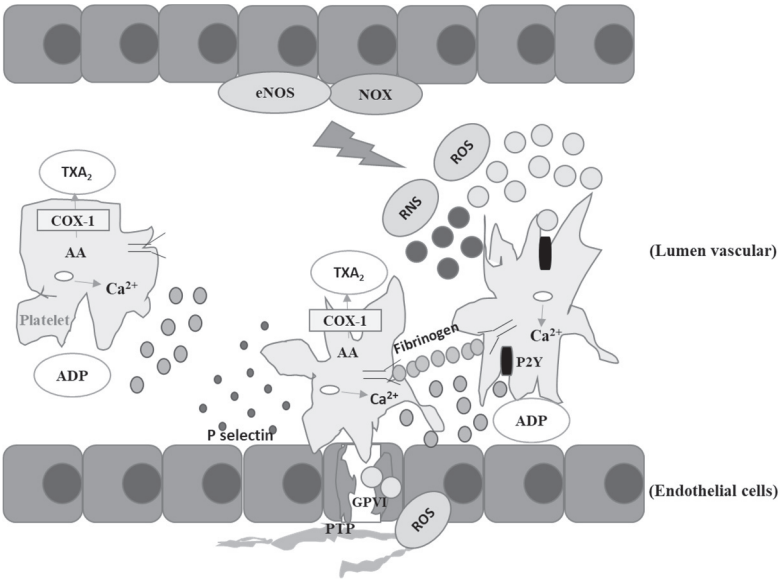

Fig. 2-1. Platelet activation after exposure to collagen and the production of free radicals.

Protein tyrosine-phosphatases (PTP) are the primary targets of ROS which, in turn, determine the upregulation of glycoprotein VI (GPVI) signalling. This event causes the overstimulation of platelets, inducing cytosolic calcium ions mobilization and the release of pro aggregating factors from granules, as ADP and TXA2. Overdue to endothelial dysfunction, eNOS and NOX stimulation lead to the overproduction of ROS and RNS which are able to interact with platelets through the ADP receptor, triggering the same intracellular signalling pathway.

(Platelet TXA2 production from arachidonic acid (AA) is dependent on COX-1 (cyclooxygenase-1); P2Y is a peptide receptor for ADP)

The role of proteases as thrombin, uPA/tPA (urokinase PA/tissue PA) or plasmin, in the pathophysiology of atherosclerosis is being put in evidence, with an unbalanced ratio between proteases and their inhibitors favouring

the chronic evolution of the plaque. Protease nexin 1 (PN-1), a serpin closed to plasminogen activator inhibitor type-1 (PAI-1), has emerged as a key regulator in vascular biology, even though its mechanism of action is hitherto unknown. Protease Nexin 1 is present in platelets and monocytes and is released from platelet α-granules during their activation. Its physiological role in coagulation, fibrinolysis and tissue remodelling and inflammation can be explained by the inhibition of a broad range of serine proteases. It displays anti-thrombotic properties via its ability to block thrombin generation and activity, and anti-fibrinolytic properties thanks to its ability to block plasmin generation and activity. PN-1 is involved in the different stages of atherosclerotic plaque progression. In the early stage, PN-1 may be involved in endothelial dysfunction and may represent a cell defence reaction against proteases present in the atherosclerotic plaque. In the advanced plaque, it is able to form covalent complexes with plasmin. Overexpression of PN-1 by VSMC has been shown to significantly reduce their adhesion, spreading and migration on vitronectin, an adhesive protein found in atherosclerotic plaques. At the most complicated stage of atherosclerosis, rupture of the plaque, platelet PN-1 is assumed to contribute to thrombus stabilization.

2.4. Endothelial layer disfunction

The arterial endothelial cells present a diversity of homeostatic functions: i) the enzymatic remodelling of extracellular matrix components; ii) the biosynthesis of vasoactive mediators, various growth factors, cytokines and hormone-like substances; iii) the enzymatic buffering of ROS, iv) the transport and metabolism of lipoproteins; and v) the synthesis of prostaglandins. When confronted with certain proinflammatory cytokines endothelial cells undergo a coordinated program of gene activation, which alters many of these vital functional properties. Endothelial layer cell dysfunction results in the earliest detectable changes in the life history of an atherosclerotic lesion, initially involving the selective recruitment of circulating monocytes from the blood to become foam cells.

An imbalance between the activity of antioxidant enzymes, such as GPx, catalase, and SOD, and the pro-oxidant system produces an uncontrolled increase in oxidative stress, leading to endothelial dysfunction. The production of oxidized LDL promotes the production of proinflammatory factors, such as interleukin-1 (IL-1), IL-6, tumour necrosis factor-α (TNF-α) and C-reactive protein (CRP) that generate the endothelial proinflammatory phenotype characterized by an increase in E-selectin,

VCAM-1 and intercellular adhesion molecule 1 (ICAM-1) expression.

Animal studies have provided compelling evidence demonstrating the roles of vascular oxidative stress and NO in atherosclerosis. ROS are also considered crucial mediators of vascular homeostasis and the atherosclerosis pathogenesis. ROS and RNS overproduction are responsible for endothelial dysregulation. Endothelial cells as well as fibroblasts and VSMC express several NOX (1, 2, 4 and 5). In particular, NOX-1 and NOX-2 are involved in the development of hypertension, inflammation, and endothelial dysfunction. The production of free radicals induced by the different isoforms of NOX influences the activity of other enzymes such as eNOS, producing NO. The endogenous production of NO in endothelial cells, at nanomolar concentrations, through NOS activation represents a vasoprotective mechanism of the vascular endothelium, while exaggerated release of NO as a consequence of a cytokine-inducible NOS activation leads, as described above, to the rapid reaction of NO with O_2^-, generating $ONOO^-$, the main compound responsible for the onset of endothelial dysfunction and, in the late stages, the development of atherothrombosis. The subsequent imbalance between vasoconstriction and vasodilatation increases the endothelial permeability and triggers a local inflammatory response. All established cardiovascular risk factors such as hypercholesterolaemia, hypertension, diabetes mellitus, and smoking enhance ROS generation and decrease endothelial NO production. Key molecular events in atherogenesis such as oxidative modification of lipoproteins and phospholipids, endothelial cell activation, and macrophage infiltration/activation are facilitated by vascular oxidative stress and inhibited by endothelial NO. Atherosclerosis develops preferentially in vascular regions with disturbed blood flow (arches, branches, and bifurcations).

2.5. Role of perivascular adipose tissue

The perivascular adipose tissue surrounds almost all the vessels with the exception of cerebral arteries, and is composed of brown or white adipocytes, or a mixture of both types, and of a stromal vascular fraction with fibroblasts, endothelial cells and immune cells. The perivascular adipose tissue has also been described as having a role in the pathophysiology of CVD, namely for modulating the vascular tone, since it presents an anticontractile function, in response to several agonists such as phenylephrine, serotonin, angiotensin II, and TXA2. In the presence of cardiovascular risk factors, such as obesity, atherosclerosis, diabetes,

hypertension and chronic alcohol consumption, oxidative stress emerges, predisposing to the vascular damage and affecting the anticontractile function of the perivascular adipose tissue. ROS are formed in the adipose tissue by different mechanisms.

In obesity, the perivascular adipose tissue inflammation and oxidative stress is caused by NOX activation, eNOS uncoupling, reduced expression of antioxidant molecules, mitochondria-derived ROS and proinflammatory cytokines. In aging and chronic alcohol consumption, ROS generation by NOX is a common feature. In response to a proatherogenic lipid profile, increased adiponectin and eNOS-derived NO is characteristic.

2.6. Autophagy

Apoptosis and autophagy are two forms of programmed cell death associated with the development of CVD. Autophagy is an intracellular catabolic mechanism for the degradation of dysfunctional proteins and organelles, essential for the maintenance of cellular homeostasis, and associated with increased longevity and health. While autophagy is characterized by the early degradation of organelles, with preservation of the cytoskeleton until the last stage, apoptosis involves the early degradation of the cytoskeleton, with preservation of the organelles until the final stage.

At the cardiovascular level, autophagy is a fundamental process for homeostasis in most cells of cardiovascular origin and for the function of the heart and vessels. Apoptosis and autophagy interact through the Beclin complex pathways (Bcl1-Bcl2/Bcl-xL), mTOR (mechanistic target of rapamycin), TRAIL (tumour necrosis factor-related apoptosis-inducing ligand), TNF-α, endoplasmic reticulum stress and p53 pathways of the nucleus. It is essential to promote cardiac and vascular health during aging, and there are some therapeutic approaches, such as pharmaceutical compounds involving mTOR inhibitor and AMPK activator to regulate apoptosis and autophagy, genetic interventions and caloric restriction, which show cardioprotective activity. Normal levels of autophagy can protect cells, however defective or excessive autophagic activity appears to contribute to cardiovascular disorders, such as heart failure or atherosclerosis.

As autophagy decreases with age, it triggers harmful cellular processes that lead to stiffening and functional decline of the arterial system. It is known that the autophagic process becomes dysfunctional as atherosclerosis progresses, even though there are, in the atherosclerotic plaque, factors that stimulate the autophagy, such as ROS. Autophagy can occur in the main

cell types of atherosclerotic plaques, that are macrophages, vascular smooth muscle cells and endothelial cells, promoting these cells survival.

Oxidized lipids, present in atherosclerotic plaques, can stimulate autophagy in macrophages. Autophagy in vascular smooth muscle cells can be initiated by atherosclerosis-related stimuli such as oxLDL, 7-ketocholesterol, TNF-α, and platelet-derived growth factor (PDGF). On the other hand, autophagy caused by factors such as osteopontin (OPN), angiotensin II and nicotine can accelerate the death of VSMC, further enhancing atherosclerotic lesions. In endothelial cells autophagic process can be stimulated by oxLDL, advanced glycation end products and saturated fatty acids. At the level of atherosclerosis, miRNA-30-mediated translational control of ATG6 regulates endothelial cell autophagy initiated by oxLDL.

However, defective autophagy can provide an accumulation of damaged proteins and organelles, such as mitochondria, resulting in increased oxidative stress and apoptosis, but accelerates stress-induced premature senescence and atherogenesis in VSMC, and in endothelial cells promotes apoptosis and also senescence. The reason for these differences is still unclear, but it may be related to increased levels of the linker molecule p62 in atherosclerotic plaques, or it may depend on the cell's origin and/or its proliferative capacity.

2.7. Inflammatory factors

Atherosclerosis results from low-grade chronic inflammation, related to unhealthy lifestyles, and also to the interaction between the immunological system and metabolic abnormalities. In a similar manner, chronic inflammatory diseases confer a significantly increase in the risk of accelerated atherosclerosis leading to enlarged morbidity and mortality and reduced life expectancy compared to the general population. In fact, there are common molecular pathways shared by atherosclerosis and inflammatory diseases. This is the case with inflammatory bowel disease, psoriasis and psoriatic arthritis, chronic obstructive pulmonary disease, rheumatoid arthritis, between others.

Patients with inflammatory bowel disease (IBD) have a higher risk of endothelial dysfunction and, consequently, of subclinical atherosclerosis than healthy controls, but the cause of the increased cardiovascular risk is not fully known. Structural changes in the arterial vessel wall occur because of long-term exposure to inflammation or cardiovascular risk factors. The pulse wave velocity levels were higher, while flow-mediated dilatation

levels were significantly decreased, in patients with Crohn's disease or ulcerative colitis relatively to healthy persons.

Psoriasis is associated with an increased risk of multiple comorbidities, including CVD at younger ages, unidentifiable by traditional risk factors. Insulin resistance appears to play a major role in the development of atherosclerosis in these cases. Patients with psoriasis show abnormalities in the innate and adaptive immune system that lead to high serum levels of proinflammatory cytokines, capable of increasing cell-mediated immunity, promoting the migration of inflammatory cells through the vascular endothelium, resulting in endothelial dysfunction and, consequently, in the formation of plaques. It seems that chronic systemic inflammation, characteristic of psoriasis and psoriatic arthritis, leads to insulin resistance, that originates endothelial dysfunction and atherosclerosis.

Chronic obstructive pulmonary disease is associated with subclinical atherosclerosis. Patients with chronic obstructive pulmonary disease seem to have a more pronounced atherosclerotic process, which is evidenced by elevated intima-media thickness, increased prevalence of carotid plaques, enlarged pulse wave velocity and reduced flow-mediated dilation. In addition, this pathology has been associated with increased levels of vascular biomarkers, regardless of physiological confounding factors, smoking or other cardiovascular risk factors. Thus, chronic obstructive pulmonary disease appears to be an independent risk factor for CVD.

Atherosclerosis, as an inflammatory process, shares mediators and activation mechanisms with rheumatoid arthritis (RA). For example, the mechanism of atherosclerotic plaque rupture has similarities with rheumatoid synovitis and destruction of joint structures. RA is associated with an increased risk of morbidity and mortality, mainly due to augmented atherosclerotic disease. RA patients evidence a higher intima-media thickness and an increased prevalence of carotid plaques.

Patients with systemic lupus erythematosus (SLE) generally have increased levels of total cholesterol, LDL, TG and apolipoprotein B (Apo B), and decreased levels of HDL, and a prevalence of metabolic syndrome of about 10%, with SLE being associated with endothelial damage and coronary atherosclerosis.

2.8. Biochemical markers of atherosclerosis

Biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. Biomarkers are characterized by elevated sensitivity, high repeatability of results and the possibility of usage in clinical procedures. They have applications in several areas, such as screening, diagnosis, prognostication, prediction of recurrences, and monitoring of therapy.

There are presently several clinical biomarkers that are related with cardiovascular outcomes. These biomarkers comprise: cardiac troponins I and T, B-type natriuretic peptides, haptoglobin, and D-dimer. Even though these biomarkers are routinely used in clinical laboratory and have helped clinicians save lives, they are late-stage biomarkers. The challenge is to find biomarkers that detect early-stage CVD to considerably reduce morbidity and mortality associated with cardiovascular effects and improve prognosis.

Recognition of classical biomarkers of atherosclerosis, such as LDL, HDL, and TG may not be effective in patients with moderate or atypical cardiovascular risk. For more precise management, non-classical atherosclerosis biomarkers may be helpful in these patients.

Biomarkers of atherosclerosis are characteristic of its phases. These comprise biomarkers of i) the inflammatory process, ii) destabilization of atherosclerotic plaque, iii) shear stress in the vascular endothelium, iv) blood vessel microcalcification, v) thrombocyte activation and vi) neurohormonal activation (Table 2-1). In the following divisions, the roles of validated biomarkers for atherosclerosis are summarized, referring on the promising candidates, the microRNAs.

Table 2-1. Main biomarkers of atherosclerosis.

Inflammatory process	Destabilization of atherosclerotic plaque	Shear stress in the vascular endothelium	Blood vessel microcalcification	Thrombocyte activation	Neurohormonal activation
Cathepsins SAA ICAM-1 VCAM-1 IL-6 TNF-α CRP MPO MMP-9 GDF15 Lp(a) ADMA	sCD40L oxLDL anti oxLDL antibody Selectins MicroRNAs PIGF PAPP-A MPO MMP-9 CRP	MicroRNAs	MicroRNAs OPN OPG	Lp-PLA2 sPLA2 sCD40L	Copeptin MR-proADM

ADMA - Asymmetric dimethylarginine; CRP - C-reactive protein; GDF15 - Growth/differentiation factor 15; ICAM-1 - Intercellular adhesion molecule 1; IL-6 - Interleukin 6; Lp(a) - Lipoprotein(a); Lp-PLA2 - Lipoprotein-associated phospholipase A2; MMP-9 - Matrix metalloproteinase 9; MPO - Myeloperoxidase; MR-proADM - Midregional pro-adrenomedullin; OPN - osteopontin; OPG – Osteoprotegerin; oxLDL - Oxidized low-density lipoprotein; PAPP-A - Pregnancy-associated plasma protein-A; PIGF - Placental growth factor; SAA - Serum amyloid-A protein; sCD40L - soluble CD40 ligand; sPLA2 - secretory phospholipase A2; TNF-α - tumour necrosis factor-alfa; VCAM-1 - vascular cell adhesion protein 1.

2.8.1. Biomarkers of the inflammatory process

Cathepsins

Cathepsins, enzymes typically concentrated in the lysosomes and endosomes of macrophages, are proteases that degrade undesirable endocytosed or intracellular proteins. Emerging research has demonstrated that cathepsins, specifically cathepsins B and X, cysteine proteases, and cathepsin D, an aspartic protease, are upregulated in atherosclerotic lesion. Another study has showed the potential of cathepsins as a diagnostic tool, having revealed that the circulating levels of cathepsin S, K and L and their endogenous inhibitor, cystatin C, could be biomarkers in the diagnosis of some pathologies, such as coronary artery disease, aneurysm, peripheral arterial disease, and coronary artery calcification. An imbalance in expression between cathepsins and its inhibitor can trigger the proteolysis of extracellular matrix in the pathogenesis of CVD. In the development phase of the disease, inflammatory cytokines, growth factors, oxidative stress, hypertensive stimuli, among others, regulate the expression and activities of cathepsins.

Serum amyloid-A protein (SAA)

SAA is an acute phase apolipoprotein that increases the expression of prothrombotic and proinflammatory molecules. Concentrations of greater than 10 mg/L were suggestively related with a greater number of new cerebral lesions detected on diffusion weighted magnetic resonance imaging during carotid artery stenting and significantly related with progressive atherosclerosis measured by ultrasound examination. Higher levels can identify patients with ischemic stroke caused by atherothrombosis *versus* cardioembolic stroke.

Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1)

Increased expression of ICAM-1 and VCAM-1 has been demonstrated in the first stage of leukocyte penetration into the vascular endothelium in vessels predisposed to the development of atherosclerosis and within existing atherosclerotic lesions. VCAM-1 and ICAM-1 are endothelial cell surface glycoproteins that allow/aid, respectively, endothelial cell-leukocyte adhesion in inflammation. The receptor for ICAM-1 is LFA-1 (CD11a/CD18, Lymphocyte function-associated antigen 1, alphaL beta2 integrin), which occurs on all types of leukocytes. The receptor for VCAM-1 is VLA-4 (CD49d/CD29, very late antigen 4, alpha4 beta1 integrin),

located on monocytes and lymphocytes. The interaction between ICAM-1/LFA-1 and VCAM-1/VLA-4 is supported by platelet endothelial cell adhesion molecule 1 (PECAM-1). This molecule transduces mechanical signals in endothelial cells and regulates migration of leukocytes under the vascular endothelium where inflammation occurs.

VCAM-1 levels have been reported to be positively linked with cardiovascular mortality, the presence of carotid atherosclerotic lesions, and magnetic resonance markers of plaque instability. There is support for a predictive function of circulating concentrations of ICAM-1 in initially healthy people and as a significant correlation was detected with cardiovascular mortality. ICAM-1 was found elevated in more than 300 patients who undertook carotid endarterectomy contrasted with healthy controls.

Interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α)

Cytokines are key regulatory glycoproteins related to inflammatory/immunological processes which modulate all aspects of vascular inflammation. IL-6 is one of the most important and most multi-functional interleukins, mainly produced by monocytes and macrophages. IL-6 enhances cell adhesion molecule expression and the production of acute phase reactants such as CRP and TNF-α. Therefore, it is associated with the development of atherosclerotic plaques. Quantifications of IL-6 may be convenient to reclassify intermediate risk patients into higher risk categories. According to the Atherosclerotic Cardiovascular Disease risk score, serum IL-6 > 1 pg/mL in patients with chest pain and intermediate risk examined for coronary angiography was predictive of major coronary artery disease. Findings on IL-6 receptors indicate that IL-6 inhibition could provide an innovative therapeutic approach to coronary heart disease prevention, but strong clinical trials and genetic testing in large populations are needed to validate and select new therapeutic targets.

On the other hand, TNF-α is a proinflammatory cytokine implicated in atherosclerotic progression from the initial phases of intimal thickening to the subsequent vessel occlusion. TNF-α is an inhibitor of endothelial nitric oxide synthase, improves the production of reactive oxygen species and reduces the effect of endothelium-derived hyperpolarizing factor. It is involved in cell differentiation and proliferation, platelet activation, and apoptosis. Senior patients who had elevated levels of TNF-α in the blood more often had clinically diagnosed atherosclerosis. TNF-α is linked with a larger plaque size and is increased in patients with plaque instability.

C-reactive protein (CRP)

CRP is a pattern recognition molecule that is elevated in inflammatory conditions, such as atherosclerosis, being an acute phase biomarker of inflammation and destabilization of atherosclerotic plaque. It belongs to the pentraxin family and is produced mainly in hepatocytes because of IL-6 stimulation. Investigations have reliably described that concentration in blood above 10 mg/L indicates an inflammatory process and higher CRP levels have a prognostic value for cardiovascular events and mortality.

CRP has been suggested to increase LDL oxidation and to induce a prothrombotic state through induction of tissue factor expression in human monocytes. It can activate or inhibit the complement cascade, driving the inflammation in atherosclerotic lesions. CRP has additionally been exhibited to reduce the expression and bioactivity of endothelial nitric oxide synthase with a subsequent effect on vasodilatation. CRP downregulates angiogenesis stimulated by vascular endothelial growth factor, while it promotes endothelial apoptosis in a nitrous oxide-dependent approach. CRP has also been found to synergistically augment angiotensin II-induced proinflammatory outcomes, involving cellular migration and proliferation as well as lesion collagen and elastin content. Ultimately, it induces the release of monocyte chemoattractant protein-1 and endothelin-1 upregulating adhesion molecules and chemoattractant chemokines in endothelial and vascular smooth muscle cells.

The high sensitivity assays (hs-CRP) quantify the low CRP concentration which cannot be assessed by the routine biochemistry analysis. A hs-CRP level of >3 mg/L should be considered as risk factor for perioperative adverse cardiovascular event in patients with asymptomatic atherosclerosis or stable ischemic heart disease and levels >10 mg/L have a greater prognostic value in those undergoing from acute coronary syndrome.

Myeloperoxidase (MPO)

MPO is a haemoprotein produced by monocytes and that activates neutrophils and catalyses the formation of hyperchlorite from chloride and H_2O_2. It promotes oxidation of LDL and oxidative modification of Apo A. MPO and metalloproteases disrupt the collagen layer in an atherosclerotic plaque, therefore leading to its erosion and rupture. Clinical trials have found that superior MPO levels are early indicators of coronary artery disease prior to detection by angiography or high cardiac troponin values.

Matrix metalloproteinase 9 (MMP-9)

Matrix metalloproteinases are zinc dependent endopeptidases produced by several cell types. Metalloproteinases are responsible for degradation of collagen and other extracellular matrix components. Matrix metalloproteinases, particularly MMP-9, are involved in all stages of atherosclerosis process. MMP-9 increases the infiltration of monocytes under the vascular endothelium. In addition, MMP-9 also influences intraplaque angiogenesis through interaction between integrins and proteinases. The aneurysm formation during atherosclerosis process is equally due to the arterial remodelling and increased extracellular matrix components breakdown by metalloproteinases.

Growth/differentiation factor 15 (GDF15)

GDF15 is produced by macrophages, cardiomyocytes, and endothelial cells in response to acute inflammatory process. The rise in GDF-15 concentrations is associated, among other conditions, to atherosclerosis, atrial fibrillation, heart failure, pulmonary embolism, and renal failure.

Lipoprotein(a) (Lp(a))

Lp(a) is a modified LDL lipoprotein by attaching a specific apolipoprotein(a) to Apo B100. The physiological functions of Lp(a) include proatherosclerotic, prothrombotic and proinflammatory roles. Lp(a) concentrations are transiently increased by inflammatory processes and by tissue damage caused by acute phase proteins, being elevated Lp(a) plasmatic level a genetically determined, independent, causative risk factor for CVD.

Alike other lipoproteins, Lp(a) is also susceptible to oxidative changes, leading to extensive formation of oxidized phospholipids, oxysterols, oxidized lipid-protein adducts in Lp(a) molecules that consolidate the progression of atherosclerotic lesions and intimal thickening by induction of M1 macrophages, inflammation, autoimmunity, and apoptosis.

Asymmetric dimethylarginine (ADMA)

ADMA is derived from the methylation of arginine residues within proteins by the activity of protein arginine N-methyltransferases. Enhanced oxidative stress upregulates this protein expression and ADMA consequently synthesis. On the other hand, pro-oxidant and proinflammatory stimuli inhibit dimethylarginine dimethylaminohydrolase activity, the enzymes responsible for ADMA degradation in cell culture research.

ADMA has been of attention to vascular researchers because this biomarker was demonstrated to be endogenous inhibitors of eNOS via competition with L-arginine, the requisite eNOS substrate and structural analogue of ADMA. Clinical data proposes that ADMA plasma concentrations are related with endothelial function, mainly in patients with coronary atherosclerosis or atherosclerosis risk factors, whereas this association is significant but weak in healthy individuals. Additionally, in prospective studies, plasma ADMA has been independently linked with clinical outcome and mortality in diabetic subjects and patients with atherosclerosis.

2.8.2. Biomarkers of atherosclerotic plaque destabilization

Circulating soluble CD40 ligand (sCD40L)

sCD40L, largely derived from activated platelets, stimulates an inflammatory reaction in vascular endothelial cells by the secretion of cytokines and chemokines. Membrane-bound CD40L and sCD40L types interact with the CD40 receptor molecule, leading to the release of MMPs and subsequent destabilization of the atherosclerotic plaque. High plasma concentrations of sCD40L were demonstrated in patients with myocardial infarction and unstable ischemic heart disease.

Oxidized low density lipoprotein (oxLDL) and anti oxLDL antibody

oxLDL can trigger the expression of adhesion molecules on the cell surface and thus promote the activation of endothelial cells. These adhesion molecules mediate the rolling and adhesion of macrophages, that adhere to the endothelium and then, in response to chemokines, migrate into the intima. As the consequence of the leukocyte activation, proinflammatory cytokines are released, ROS are synthesized, and MMPs are also produced, contributing to the matrix degradation. OxLDL also induces apoptosis of smooth muscle cells. Furthermore, oxLDL impairs nitric oxide production in endothelial cells. This leads to atherosclerotic plaque destabilization and rupture.

Apart from the scavenger receptors, oxLDL also binds to lectin-like oxidized low density lipoprotein receptor 1 (LOX-1). Later, the same receptor was shown on the surface of smooth muscle cells and macrophages.

The oxLDL molecule acts as an antigen leading to the production of anti oxLDL antibodies. LDL oxidation can affect different parts of its molecule,

which is the reason why different anti-oxLDL antibodies can be produced. IgM anti-oxLDL antibodies have been shown to reduce the risk of severe coronary artery disease. In the case of IgG anti-oxLDL class, this compound is more complex and needs additional investigation.

Selectins

Selectins are a group of cell-surface glycoproteins involved in the rolling and anchoring of leukocytes on the vascular wall. For instance, L-selectin is expressed on all granulocytes and monocytes and on most lymphocytes. It has been associated to larger plaque size estimated by ultrasound imaging in patients with carotid atherosclerotic plaques. Also, the important function of P-selectin in both leukocyte recruitment and atherosclerosis progression has been confirmed in various animal models and several authors have confirmed that a deficiency of this adhesion molecule has a protective result against atherosclerosis and an increase level is associated with progression of atherosclerosis, coronary artery disease, and atrial fibrillation.

MicroRNAs (miRNAs or miRs)

MicroRNAs are short, non-coding segments of RNA containing 18-26 nucleotides that function to silence mRNAs and thus prevent the translation of messenger RNAs (mRNAs) to proteins. They relocate from one cell to another in a process of intercellular communication to silence specific mRNAs in the target cell. miRNAs distribute in the body in membrane-derived vesicles, such as micro vesicles, exosomes, and apoptotic bodies, as well as bound to RNA-binding proteins, or by HDL cholesterol. The function of miRNAs has been recently proposed as next generation biomarkers due to their integral role in mediating cellular and molecular roles. miRNAs are involved in the pathogenesis of many diseases. Selected miRNAs implicated in the development of the atherosclerotic formation/rupture are shown in the Table 2-2.

Table 2-2. miRNAs involved in atherosclerotic process - most important examples.

miRNAs	Function
Plaque inflammation	
miRNA-126	Inhibition of VCAM-1
miRNA-155, -222, -424, -503, -9, -17, -20a, -106a	Regulation of monocyte differentiation into macrophages within the plaque
miRNA-147, -155, and -342-5p	Stimulation of plaque macrophages in M1 phenotype; Up-regulation of TNF-α and IL-6 cytokines
miRNA-125a, -146a, -33, and -155	Inhibition of lipid agglomeration
miRNA-15a, -16 s	Modulation of macrophage apoptosis
miRNA-21, miRNA-34a	Synthesis of MMP-9; VSMC proliferation
miRNA-210	Linked to intraplaque angiogenesis and, possibly, to the formation of unstable plaques; regulates endothelial apoptosis
miRNA-146a	Formation of the T helper type-1 mononuclear phenotype
miRNA-29	Inhibition of elastin expression
miRNA-221/222	Stimulation of cell proliferation or apoptosis
miRNA-365	Stimulation of endothelial cells apoptosis
miRNA-100, -127, -145, -133a, -133b	High-level expression in symptomatic carotid plaque
Endothelial shear stress	
miRNA-143 and 145	Development of VSMC transfer into the atheroprotective contractile phenotype
miRNA-126-5p	Limitation of endothelial cell proliferation at sites of low endothelial shear stress
miRNA-92a	Related with low endothelial shear stress, expansion of inflammation
Microcalcification	
miRNA-29a and miRNA-29b	Suppression of the disintegrin and metalloproteinase expression.
miR-125b	Differentiation of VSMC into an osteoblast-like phenotype

The most important advantage of employing miRNAs as biomarkers is the opportunity for assessment of selected miRNAs by using the standard technology for the detection and/or comparison of RNA concentrations (the quantitative reverse transcriptase-polymerase chain reaction method), with great sensitivity and specificity. However, prospective large-scale human studies are required to authenticate the real potential of circulating miRNAs and changes in miRNA expression; hence, circulating miRNAs can serve as independent biomarkers of atherosclerotic diseases, and, moreover, whether other more readily accessible body fluids, such as urine or saliva, may be suitable for diagnosis.

Placental growth factor (PIGF)

PIGF is a growth factor that belongs to the family of endothelial growth factor. It plays an important role in the pathogenesis of atherosclerosis by stimulating angiogenesis and increasing the migration of monocytes and macrophages into the vascular endothelium, which subsequently produces inflammatory and vascular mediators, resulting in an increased risk of plaque rupture. It has been shown that overweight children and with the metabolic syndrome have higher PIGF levels in the blood compared to healthy children. In addition, a positive correlation was found between PIGF and troponin plasmatic concentrations.

Pregnancy-associated plasma protein-A (PAPP-A)

PAPP-A, a metalloproteinase produced by the placenta, can enhance local insulin-like growth factor (IGF) bioavailability through proteolytic cleavage of three IGF binding proteins events. This enzyme exerts a proatherogenic effect by altering a variety of pathological processes implicated in atherosclerosis, including lipid accumulation, vascular inflammation, endothelial dysfunction, vascular smooth muscle cell proliferation and migration, plaque stability, and thrombus formation. In patients with coronary atherosclerosis disease, increased PAPP-A levels are significantly associated with a higher risk of CVDs.

MPO, MMP-9 and CRP

Biomarkers of atherosclerotic plaque destabilization similarly include MPO, MMP-9 and CRP described above.

2.8.3. Biomarkers of shear stress in the vascular endothelium

A variety of miRNAs are included as shear stress biomarkers. For instance, and as showed in Table 3.2, microRNA-143 and microRNA-145 switch the phenotype of VSMC to contractile ones. microRNA-126-5p restricts the proliferation of vascular endothelial cells, while microRNA-92a improves the development of inflammatory processes in the vascular wall.

2.8.4. Biomarkers of blood vessel microcalcification

microRNAs

Vascular calcification is a prominent aspect of atherosclerosis, and some miRNAs are involved. microRNA-29a and microRNA-29b inhibited calcification of VSMC by suppressing the expression of a disintegrin and metalloproteinase with thrombospondin motifs 7. Moreover, miR-125b downregulation can promote calcification of vascular smooth muscle cells by targeting Ets1, a transcription factor protein. Furthermore, VSMC trans-differentiation into osteoblast-like cells can be promoted by the inhibition of endogenous miR-125b with the osteoblast transcription factor SP7, as its target, which can regulate osteoblast differentiation.

Osteopontin (OPN) and osteoprotegerin (OPG)

A growing number of stimulatory and inhibitory molecules imply that vascular calcification is an actively controlled process. Among these molecules OPN, an acidic phosphoprotein, and OPG, a member of the TNF-α receptor super family, have been proved to inhibit mineral deposition as well as osteoclast genesis and they are constitutively expressed by an extensive range of cell types in the vasculature

These bone-matrix proteins, which attenuate vascular microcalcification, are biomarkers of atherosclerotic plaque composition and CVD prognosis. Data derived from clinical investigations support the notion that increased serum levels of this markers are positively associated with acute cardiovascular events, coronary disease severity and poor long-term cardiovascular results. Circulating OPN-OPG amounts were higher in patients bearing carotid stenosis with unstable atherosclerotic and in symptomatic *versus* asymptomatic patients, who presented superior calcification.

2.8.5. Biomarkers of thrombocyte activation

Lipoprotein-associated phospholipase A2 (Lp-PLA2) and secretory phospholipase A2 (sPLA2)

Lp-PLA2 is a proinflammatory protein produced by monocytes, lymphocytes, and mast cells; 80% is bound to LDL cholesterol, and 20% to HDL cholesterol. Numerous studies have demonstrated that Lp-PLA2 plays a causal role in atherosclerosis and inhibiting Lp-PLA2 improves vascular inflammation and decelerates the progression of atherosclerosis. Additionally, several meta-analyses and epidemiological studies and have also reliably proven that increased plasma levels of Lp-PLA2 are associated with an increased risk of cardiovascular events. sPLA2 is the phospholipase A2 isozyme and hydrolyses the sn-2 ester bond in glyceroacyl phospholipids of lipoproteins and cell membranes, producing non-esterified fatty acids and bioactive lysophospholipids implicated in acute and chronic inflammatory processes. Expression of sPLA2 is up-regulated in response to cytokines such as interferon-γ, TNF-α, IL-1β, and oxLDL. The relationship between sPLA2 concentration and CVD prospect was properly demonstrated. For example, in coronary artery disease patients, an increase in circulating sPLA2 levels is a significant risk factor of clinical coronary events during follow-up.

sCD40L

The biomarkers of thrombocyte activation also include the previously described sCD40L.

2.8.6. Biomarkers of neurohormonal activation

Copeptin

Copeptin is secreted from the posterior pituitary gland into the circulation in stoichiometric amounts along with vasopressin. Both neuropeptides are primarily released in response to hemodynamic or osmotic changes. In contrast with vasopressin, copeptin exhibits higher plasma and serum stability, allowing its use in laboratory diagnostics. After release, it remains stable for several days and copeptin levels increase rapidly in conditions, such as CVD, stroke, sepsis, and shock. Some researchers have been demonstrated that the increased concentration of copeptin in blood positively correlates with the risk of developing coronary heart disease and the risk of death due to CVD.

Midregional pro-adrenomedullin (MR-proADM)

MR-proADM is a stable and surrogate measure for mature adrenomedullin (ADM), and provides useful information, particularly in the short-term. ADM is a peptide hormone produced by the adrenal medulla, heart, and vascular endothelial cells that acts as a vasodilator and plays important roles in the microcirculation and in endothelial dysfunction. ADM has some cardiovascular actions, including those promoting vasorelaxation, natriuresis and increasing cardiac output. A potential role of ADM in calcification processes in the heart and aorta has also been shown in animal and cell culture studies. Immunoluminometric assays for the measurement of the MR-proADM precursor fragment in human plasma have been reported, thus making it a promising biomarker of the risk of developing coronary heart disease and heart failure. Additionally, MR-proADM may be a prognostic biomarker after ST-elevation myocardial infarction.

2.9. Treatment of atherosclerosis

Dyslipidaemia, and particularly hypercholesterolaemia, is considered the main cause of atherosclerosis, with LDL-c, TG, and HDL-c as strong independent predictors of atherosclerotic disease after the analysis of the data from the Framingham study. The high level of HDL-c is considered a protective parameter, while low HDL-c has been shown to be a strong independent predictor of premature atherosclerosis.

Lp(a) is a specialised form of LDL, consisting of an LDL-like particle and the specific ApoA. Its elevation in plasma is an additional independent risk marker and can be related to the pathophysiology of atherosclerotic vascular disease and aortic stenosis.

The control of dyslipidaemia is the cornerstone of prevention and treatment of atherosclerosis, and can be achieved by lifestyle modifications, eventually with the use of specific lipid lowering drugs.

Non-pharmacological measures

Measures such as dietary changes, weight control, stimulation of physical activity and smoking and alcohol eviction, preferably to be implemented since infancy, are effective for the prevention and control of dyslipidaemia.

Pharmacological measures

Medication to adequately control lipoprotein levels needs to be initiated when the plasma lipid values are altered, after a variable period of lifestyle modifications and depending on the global cardiovascular risk of the patient. In secondary prevention, medical therapy is almost invariably needed in addition to lifestyle optimisation.

Statins (3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors) induce an increased expression of LDL receptors (LDL-r) on the surface of the hepatocytes, decreasing plasma concentration of LDL and other Apo B-containing lipoproteins, including TG-rich particles (although benefits of lowering elevated TG levels are modest for reducing cardiovascular risk). Statins can also elevate HDL-C levels between 5-10%.

The most used cholesterol absorption inhibitor is ezetimibe. It is usually used in combination with statins, or in monotherapy when statins are not tolerated. In monotherapy, ezetimibe can reduce LDL by 15-22% and when combined with a statin it induced an incremental reduction in LDL levels of 15-20%.

Bile acid sequestrants (cholestyramine, colestipol) can produce a reduction in LDL of 18-25%, but their use is limited by gastrointestinal adverse effects and major drug interactions with other frequently prescribed medications.

Proprotein convertase subtilisin/kexin type 9 inhibitors (PCSK-9 inhibitors), evolocumab and alirocumab, two monoclonal antibodies that inhibit PCSK-9, offer the prospect of achieving even lower LDL levels than statins in combination with ezetimibe. PCSK-9 stimulates the absorption and degradation of the receptors for LDL in the hepatocytes. Through inhibition of PCSK-9, the degradation of LDL-r is prevented, improving the absorption of LDL particles and lowering LDL plasma concentrations. In clinical trials, the PCSK-9 therapy lowered LDL by 50% and demonstrated a significant percentage atheroma volume decrease. The PCSK-9 therapy is suitable in a wide range of patients provided that they express the LDL receptor, including those with heterozygous and homozygous familial hypercholesterolaemia.

Fibrates are agonists of peroxisome proliferator-activated receptor-α (PPAR-α); they are effective in lowering fasting TG, post-prandial TGs and TG-rich lipoprotein remnant particles, lowering TG levels up to more than 50%. Fibrates increase HDL-c in a similar proportion with statins, namely between 5% and 15%.

n-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) can lower TG up to 45%, possibly through interaction with PPAR.

Cholesteryl ester transfer protein (CETP) inhibitors, a new class of pharmacologically active molecules, seems to be effective in increasing HDL-c levels, with an effect of ≥ 100% increase in HDL-c and frequently a reduction of LDL-c levels as well.

Conclusion

Atherosclerosis is a pathologic process by the accumulation of lipids in the plasma and its deposition on the cell wall triggers a cascade of events leading to vascular wall thickening, luminal stenosis, calcification, and thrombosis. This process leads to coronary artery disease and myocardial infarction, carotid artery stenosis and stroke, abdominal aortic aneurysms, peripheral vascular disease with lower-extremity claudication and, in some cases, death.

ROS are significant contributors to atherosclerosis, causing oxidative modification of LDL. ROS can also promote endothelial dysfunction and a vascular inflammatory response. Excessive oxLDL and the upregulation of LOX-1 expression can lead to defective autophagic mechanisms and can trigger inflammatory and oxidative stress responses. Proinflammatory factors, such as IL-1, IL-6, TNF-α and CRP generate the endothelial proinflammatory phenotype and endothelial cells dysfunction. Moreover, there is a prothrombotic effect of vascular-derived and platelet-derived ROS and an outcome over the perivascular adipose tissue, affecting its anticontractile function, predisposing to vascular damage.

Classical biomarkers of atherosclerosis, such as LDL, HDL, and TG are suitable for the average patient, but may not be effective in patients with moderate or atypical cardiovascular risk. Other biomarkers are under study, comprising biomarkers of the inflammatory process, destabilization of atherosclerotic plaque, shear stress, microcalcifications, thrombocyte activation and neurohormonal activation, and the promising candidates, the microRNAs.

Treatment of atherosclerotic is based on lowering LDL by statin therapy. In high-risk patients with statin intolerance or who do not obtain the target level of LDL, association with ezetimibe, or other drugs should be considered. PCSK-9 inhibitors are used in hypercholesterolaemia patients that do not respond satisfactorily to other therapies.

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Chapter 3

Biochemical Changes in Musculoskeletal Diseases

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List of abbreviations

ADAMTS: A disintegrin-like and metalloproteinase with thrombospondin
AMP: Adenosine monophosphate
ATP: Adenosine triphosphate
BMD: Bone mineral density
BMI: Body mass index
CoA: Coenzyme A
CPT: Carnitine palmitoyltransferase
CTX-I: C-terminal telopeptide I, carboxy-terminal collagen I crosslinks
DNA: Deoxyribonucleic acid
DPD: Deoxypyridinoline
GM-CSF: Granulocyte macrophage-colony stimulating factor
GMP: Guanosine monophosphate
IL: Interleukin
M-CSF: Macrophage-colony stimulating factor
MMPs: Metalloproteinases
NTX-I: N-terminal telopeptide I, carboxy-terminal collagen I crosslinks

WHO: World Health Organisation
PGE2: Prostaglandin E2
PICP: Procollagen type I C-terminal propeptide
PID: Pyridinoline
PINP: Procollagen type I N-terminal propeptide
PRPP: Phosphoribosyl pyrophosphate
PTH: Parathyroid hormone
RNA: Ribonucleic acid
TGFβ: Transforming growth factor β
TNF: Tumour necrosis factor
TRAP: Tartrate-resistant acidic phosphatase

Introduction

When considering musculoskeletal diseases, more than 150 possible diagnoses of conditions that affect the locomotor system should be considered. These conditions are characterised by pain, decreased physical abilities, big impacts in mental health and are a strong cause for the development of other chronic diseases such as obesity, since sedentarism and often incorrect food intake are associated. Musculoskeletal diseases have a big social and economic impact. Most patients require premature retirement and extremely expensive health care for long periods of time.

Pathological conditions that affect the musculoskeletal system are commonly related with older ages. However, the development of pain in the back, neck and lumbar region has been increasing in children, adolescents, and middle-aged adults. There is no aetiology that underlies all musculoskeletal disorders, being often of multifactorial origin. Psychosocial factors, such as overwork, are usually related to the evolution of these conditions.

The World Health Organization (WHO) considers musculoskeletal diseases the epidemic of the 21st century. They have a great impact on society and economy. The early diagnosis of these conditions and the adoption of effective preventive measures are imperative.

In this chapter, musculoskeletal disorders will be divided into three subgroups according to the affected organ or tissue. Thus, bone, joint and muscle diseases will be addressed.

3.1. Bone diseases

Bone is a reservoir of minerals and performs important functions in the body, such as providing support for the body, allowing locomotion, protecting organs, ensuring attachment sites for the muscular system, and sustaining the force of muscle contraction. Bone is based on a hardened mineralised matrix to allow this tissue to perform its support functions. Bone can be classified in two ways: compact bone, important for the development of support functions and mechanical protection; and cancellous bone, with a honeycomb-like structure, representing a site for the formation of new bone cells and a reservoir of minerals.

3.1.1. Bone metabolism

Bone metabolism is continuous, dynamic, and cyclic, maintaining a balance between bone resorption and formation, and is responsible for maintaining calcium homeostasis, acid-base balance, and release of growth factors.

Bone metabolism involves the action of osteoclasts in bone resorption, being important at the beginning of the bone remodelling cycle; of osteoblasts involved in bone formation; and of osteocytes with a preponderant role in bone maintenance. Osteocytes, the most abundant cells, support a three-dimensional interconnected network throughout the bone, acting as stress sensors, reacting to exogenous factors leading, for example, to the formation of new osteocytes.

After apoptosis of osteocytes, multicellular bone units are recruited to the site. Each of these units consists of different cell types with different morphologies and functions such as osteocytes, osteoclasts and osteoblasts that act in a coordinated manner in the bone remodelling compartment.

At an early stage, osteoblasts are inhibited by osteocytes. These cells promote the recruitment of osteoclast precursors from the bone marrow, which differentiate into mature osteoclasts that produce hydrogen ions, lactate, and proteolytic enzymes, which induce the degradation of bone tissue and the release of calcium and other matrix constituents, instigating the formation of cutting cones.

The osteoblasts advance on the weakened bone by the cutting cones and start the formation of new bone tissue over the cavity. Thus, an osteoid matrix is secreted which serves as a precursor of new bone formation. Some osteoblasts remain attached to the matrix and differentiate into osteocytes.

The new bone is like the old one, ensuring that no change occurs in bone mass. Bone renewal only occurs when necessary and to the desired extent, being regulated by endogenous and exogenous factors such as electrical and mechanical forces, hormones, vitamin D, oestrogens, androgens, cortisol, growth factors and cytokines. Changes in the balance of this cycle caused by ageing, metabolic diseases, mobility alterations and certain therapies can trigger pathologies such as rickets, osteomalacia, osteoporosis, Paget's disease, among others.

3.1.2. Biochemical markers of bone remodelling

Biochemical markers of bone renewal allow a clarified and real time evaluation, and they can be measured in blood or urine. Three categories of biochemical markers can be defined, (a) enzymes or proteins secreted by the cells involved in the bone renewal process; (b) degradation products produced in bone resorption; and (c) by-products produced during the synthesis of new bone.

Considering the bone renewal cycle, two types of biomarkers must be considered: those with an active role in bone resorption and those which act in bone tissue formation.

Biochemical markers of bone resorption

These biochemical markers are the enzyme tartrate-resistant acidic phosphatase (TRAP) and the products of collagen catabolism (Table 3-1).

TRAP is a lysosomal enzyme found mainly in bone. TRAP present in osteoclasts is the most unstable, losing activity with changes in temperature. The presence of this enzyme can be measured in serum or plasma, and higher levels are likely to be found in serum as it is secreted by platelets during the clotting process. Since the blood level of this enzyme is not affected by renal function, it is considered a useful marker of bone resorption in patients with renal failure.

During bone resorption, osteoclasts promote collagen degradation, leading to the formation of the N-terminal cross-linking telopeptide of type I collagen (NTX-I) and the C-terminal cross-linking telopeptide of type I

collagen (CTX-I). These peptides, resulting from the degradation of collagen, are transported in the bloodstream, and partly excreted in the urine. Part of NTX-I and CTX-I is degraded to pyridinoline (PID) and deoxypyridinoline (DPD) which are also excreted in the urine.

CTX-I can be measured in serum, plasma and urine and should be collected while fasting, since its levels are affected by the circadian rhythm and are highest in the morning. NTX-I is measured in serum and urine. It is determined by an immunological assay. The anti-NTX-I antibody is not specific for the epitope of bone type I collagen and therefore CTX-I is more specific. DPD is also more specific for bone compared to PID which is present in cartilage and ligaments, which decreases its specificity. Both DPD and PID are measured in urine.

Biochemical markers of bone formation

Biochemical markers of bone formation include the enzyme alkaline phosphatase, osteocalcin, and type I collagen propeptides (Table 3-1).

Alkaline phosphatase comes in different isoforms and can be found in the liver, intestine and placenta. During adulthood it is the bone and liver isoforms that contribute to the assessment of total alkaline phosphatase. Three genes coding for alkaline phosphatase exist in chromosome 2 that are expressed in a tissue-specific manner, being present in placenta and intestine. A fourth gene is present in chromosome 1 and encodes a family of proteins. These isoenzymes only differ from each other by post-translational modifications and are most abundant in hepatic, renal and skeletal tissues.

Osteocalcin, a protein composed of 49 amino acids, can be found in bone and dentin. Because it is synthesised by osteoblasts and odontoblasts, its serious levels are bone specific. Osteocalcin is quantified in serum or plasma samples and can be found intact or a fragment resulting from its degradation in the blood circulation. The antibodies used to determine osteocalcin recognise both the intact molecule and the fragment. Osteocalcin is degraded and excreted by the kidney, so its concentrations may be increased in patients with renal insufficiency. Vitamin K stimulates the carboxylation of osteocalcin, allowing this carboxylated form to identify fractures.

Last are the pro-collagen type I molecules, produced by osteoblasts, which undergo cleavage at the amino and carboxyl termini forming N-terminal pro-collagen type I peptide (PINP) and C-terminal procollagen type I

peptide (PICP), respectively. Both incorporate the bone matrix and are excreted by the liver. Their determination presupposes the application of immunological assays.

Type I collagen is not a protein unique to bone tissue. However, circulating PINP and PICP come essentially from bone, since type I collagen in the body is essentially found in bone, and the skeleton is relatively heavier than other tissues. PINP is more sensitive when compared to PICP.

Biochemical markers have been shown to be important in determining changes in metabolism in bone tissue. All markers may be relevant for any alteration, but those with greater specificity for bone tissue are the ones that prove to be decisive for the diagnosis of any bone pathology.

Table 3-1. Biochemical markers of bone renewal.

Biochemical marker		Tissue	Sample	Observations
Resorption				
TRAP		Bone; blood	Serum and plasma	Has six isoforms
Hydroxyproline		Bone; cartilage; tendons; soft tissue; skin	Urine	Present in tissues with type I collagen
Products resulting from collagen catabolism	**NTX-I**	Bone; cartilage; tendons; soft tissues; skin	Serum and urine	Present in tissues with type I collagen
	CTX-I	Bone; cartilage; tendons; soft tissues; skin	Serum, plasma, and urine	Present in tissues with type I collagen; higher specificity for collagen present in bone
	PID	Bone; cartilage; tendons; soft tissues; skin	Serum and urine	Present in tissues with type I collagen
	DPD	Bone and dentin	Serum and urine	Present in tissues with type I collagen; higher specificity for collagen present in bone

Formation			
Alkaline phosphatase	Bone; liver; intestine	Serum	Presents several isoforms
Osteocalcin	Bone; dentin; platelets	Serum and plasma	Secreted by osteoblasts; selective marker for bone reabsorption
PINP	Bone; soft tissues; skin	Serum	Secreted by osteoblasts and fibroblasts; incorporates the bone matrix
PICP	Bone; soft tissues; skin	Serum	Secreted by osteoblasts and fibroblasts

3.1.3. Rickets

According to the literature, rickets is the non-contagious disease that affects the largest number of children worldwide. This pathology is defined as a bone disease associated with decreased serum calcium and/or phosphate levels.

Rickets leads to the development of bone deformities due to increased and delayed mineralisation of the growth plates which essentially affect the lower limbs. These can evolve into bowed legs, alterations in the knees, pelvic deformities and, in more serious cases, predisposition to a greater occurrence of fractures. Apart from bone problems, rickets can also lead to the development of respiratory tract infections, convulsions, and recurrent gastroenteritis.

The diagnosis of this pathology should be as early as possible to start treatment before the age of eight months. This early diagnosis is essential to minimise the morbidity caused by this pathology. The increased prevalence of this disease in developed and developing countries is linked to several factors that include diets low in calcium and vitamin D, decreased sunlight exposure, decreased vitamin D and calcium in pregnant and breastfeeding women.

Rickets develops because of vitamin D deficiency. This vitamin is essential for maintaining calcium homeostasis and for the immune system, with relevance in preventing autoimmune diseases, cardiovascular diseases, cancer, asthma, and allergies.

Vitamin D obtained from diet or sun exposure is converted in the liver into 25-hydroxyvitamin D by the enzyme 25-hydroxylase. 25-hydroxyvitamin D is then converted to 1,25-hydroxyvitamin D by the enzyme 1α-hydroxylase in the kidneys. 1,25-Hydroxyvitamin D mediates the absorption of calcium and phosphate at the gastrointestinal level to allow their incorporation into bone. Conversion to 1,25-hydroxyvitamin D is influenced by parathyroid hormone or parathormone (PTH).

A decrease in circulating vitamin D is associated with decreased calcium and phosphate absorption at the intestinal level. This leads to increased levels of PTH, inducing increased renal reabsorption of calcium and decreased phosphate.

Types of rickets

The literature classifies this pathology into nutritional rickets, calcipenic rickets and phosphopenic rickets (Table 3-2).

Nutritional rickets is related to low intake of calcium, phosphate and vitamin D and inadequate sun exposure.

Calcipenic or vitamin D-dependent rickets is associated with vitamin D or calcium deficiency, either as a nutritional condition or due to genetic mutations. A mutation in the CYP2R1 gene leads to 25-hydroxyvitamin D deficiency, an inherited condition also known as vitamin D-dependent rickets type 1B. A mutation in the CYP27B1 gene, or kidney disease, induces 1,25-hydroxyvitamin D deficiency (vitamin D-dependent rickets type 1A). A mutation in the VDR gene (vitamin D-dependent rickets type 2), decreases affinity for the active metabolite, 1,25-hydroxyvitamin D. Treatment of this condition essentially involves oral administration of the active form of vitamin D, cholecalciferol or ergocalciferol.

Phosphopenic rickets involves a decrease in the activity of phosphate cotransporters in the renal proximal tubules which leads to a decrease in phosphate reabsorption and its consequent elimination in the urine. In this condition plasma phosphate levels are decreased, but the same is not true of 25-hydroxyvitamin D and PTH, which are at levels considered normal. This form of rickets is hereditary due to a mutation in the phosphate regulator gene on the X chromosome. Phosphopenia leads to normal mineralisation of the growth plates and its treatment involves oral administration of phosphate in concomitance with the active form of vitamin D.

Table 3-2. Types of rickets.

Types of rickets		Causes
Nutritional rickets		• Diet low in calcium, phosphate, and/or vitamin D • Low sunlight exposure
Calcipenic rickets	Rickets dependent on type 1B vitamin D	• Mutations in *CYP2R1* gene
	Rickets dependent on type 1A vitamin D	• Mutations in *CYP27B1* gene
	Rickets dependent on type 2 vitamin D	• Mutations in *VDR* gene
Phosphopenic rickets		• Mutation in the gene regulating phosphate (present in the X chromosome)

Biochemical markers in rickets diagnosis

Different biochemical aspects have been considered for the diagnosis of rickets. Some biochemical alterations are more relevant than others according to the type of rickets. The biochemical markers used are essentially calcium, phosphate, alkaline phosphatase, PTH, 25-hydroxyvitamin D and the calcium/creatinine ratio in urine, which makes it possible to establish calcium excretion. Often, for lack of means, only serum calcium, phosphate and alkaline phosphatase levels are considered.

Alkaline phosphatase values are found to be high in all types of rickets, with calcipenic rickets typically showing higher values when compared to those obtained in an individual with phosphopenic rickets. According to the literature, alkaline phosphatase is the most used biochemical marker for the diagnosis of rickets, but elevated values of this enzyme may also indicate other pathological conditions. Therefore, serum alkaline phosphatase activity is also used as a biochemical marker for the diagnosis of other pathologies such as primary hyperparathyroidism, osteomalacia and Paget's disease.

PTH values are elevated in cases of calcific rickets and contrast with normal levels in cases of phosphopenic rickets. The increase in PTH hormone in calcific rickets reflects the increase in alkaline phosphatase which induces an intensification of bone turnover. This is particularly important when monitoring therapy since its levels reduce significantly because of adequate and effective treatment.

Vitamin D deficiency is confirmed by measuring 25-hydroxyvitamin D. When it is low, the condition of rickets is confirmed. Analysis of 1,25-hydroxyvitamin D is only performed in cases of suspected mutations in vitamin D metabolism and then confirmed by genetic testing.

3.1.4. Osteoporosis

Osteoporosis is a pathology characterised by a decrease in bone mass, which affects mainly postmenopausal women. Osteoporosis is a bone disease in which the metabolism of the bone is altered, leading to decreased bone mass with deterioration of the microarchitecture of the bone, resulting in increased bone fragility and a propensity to develop fractures.

According to the WHO, osteoporosis is defined by bone mineral density (BMD) in the hip or lumbar region, being considered present for values less than or equal to 2.5 standard deviations of the average BMD value of a reference population composed of young adults.

Among the various factors contributing to a decrease in BMD levels are calcium and vitamin D deficiencies, the marked decrease in oestrogens after menopause and the increase in serum PTH levels, these being the most prevalent factors in the development of osteoporosis. As with rickets, the main reasons for low vitamin D levels are poor sun exposure and poor diet. A decrease in vitamin D levels implies a decrease in calcium absorption (Fig. 3-1).

Regarding the bone metabolism mentioned above, it is known that during bone resorption there are processes of matrix catabolism where cavities are formed. These are compensated by osteoblasts that fill these cavities with bone matrix formation. In osteoporosis there is an imbalance between bone resorption and bone formation.

The clinical sequela with the greatest impact of this disease is the increased risk of fracture, the most common being fractures of the vertebrae, the proximal femur (hip) and the distal forearm. Many of these fractures are accompanied by chronic pain and can lead to patient disability. Data from 2011 indicate that in the European Union osteoporosis affects about 22 million women and 5.5 million men with alarming data regarding the number of fractures: 610,000 hip fractures, 520,000 vertebral fractures, 560,000 forearm fractures and 1,800,000 other fractures.

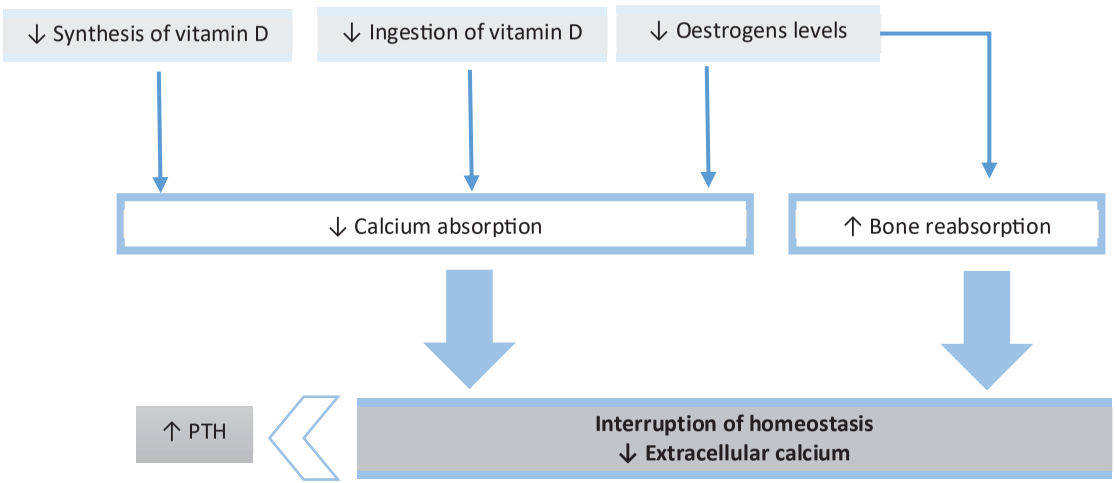

Fig. 3-1. Physiopathology of osteoporosis.

Biochemical markers in osteoporosis diagnosis

Recommendations for the diagnosis of osteoporosis are essentially based on the measurement of BMD by dual-energy X-ray absorptiometry of the lumbar spine or of the femoral neck, the results being expressed in T-index (Table 3-3). The T-index is understood as the expression in standard deviations of the BMD of the individual under study compared to the BMD of a young group of the same sex, corresponding to the age group at the peak of bone mass.

Table 3-3. Criteria used in the diagnosis of osteoporosis.

Criteria of diagnosis expressed in standard deviation of BMD	Classification
T ≥ -1	Normal
-2,5 < T < -1	Osteopenia
T ≤ -2,5	Osteoporosis
T ≤ -2,5 with fracture	Strong osteoporosis

Individuals who can be nominated for densitometry are those with one major, or two minor risk factors for osteoporosis (Table 3-4); women over 65 years of age; or men over 70 years of age.

For the diagnosis of osteoporosis, in addition to the evaluation of BMD measured by bone densitometry, it is also important to know the patient's clinical history. There are several risk factors that contribute to the development of this condition, such as advanced age, female gender, being Caucasian, previous fracture, family history of fracture, low body mass index (BMI), treatment with corticosteroids, smoking, alcohol abuse, physical inactivity, low calcium diet and chronic inflammatory diseases such as rheumatoid arthritis (Table 3-4). Biochemical markers are of considerable importance in osteoporosis and are widely used to assess response to therapy and predict possible fractures.

The most relevant biochemical markers in osteoporosis are CTX and PINP, with CTX being used to assess bone resorption and PINP providing information on formation. Although these biomarkers allow assessment of the balance between bone turnover and bone formation, they are not strongly related to BMD. As they have little specificity for bone tissue and vary widely with factors such as age and gender, they are not used for the diagnosis of the disease.

Table 3-4. Risk factors for the development of osteoporosis.

Major **risk factors**	***Minor*** **risk factors**
Previous spinal fracture	Rheumatoid arthritis
Fragility fracture after the age of 40	History of clinical hyperthyroidism
History of hip fracture in one parent	Chronic treatment with anti-epileptics
Systemic corticoid therapy of more than 3 months duration	Low calcium intake in the diet
Premature menopause	Smoking
Hypogonadism	Excessive caffeine consumption (more than two cups a day)
Primary hyperparathyroidism	Excessive consumption of alcoholic drinks
Increased risk of falling	BMI lower than 19 kg/m^2
	Weight loss of more than 10% in relation to the individual's weight at age 25
	Chronic therapy with heparin
	Long-term immobilisation

3.1.5. Osteomalacia

Osteomalacia is one of the bone diseases with the least impact on the world, unlike osteoporosis which affects a large percentage of the population, especially females. This pathology is characterised by deficient bone mineralisation resulting from hypocalcaemia and hypophosphatemia, due to a low concentration of vitamin D.

As previously mentioned, vitamin D plays a fundamental role in bone mineralisation. Low levels of vitamin D may be at the origin of most cases of osteomalacia and therefore these can be resolved with measures leading to an increase in serum levels of it. However, it is now clear that many cases of osteomalacia cannot be resolved by increasing vitamin D.

Osteomalacia manifests itself through vague and unspecific symptoms. Patients usually present with pain, muscle weakness, fatigue and motor difficulties that diminish during the summer months. Faced with this symptomatology, it is likely to incur a misdiagnosis as these symptoms fall under several diseases, including fibromyalgia, severe myopathy, uncommon pain syndrome or neurological disorders.

Biochemical markers in osteomalacia diagnosis

When osteomalacia is suspected there are some biochemical markers that should be evaluated. Serum calcium and phosphate, PTH, 25-hydroxyvitamin D and alkaline phosphatase levels, besides evaluation of renal function are essential parameters for a correct diagnosis of this pathology. Osteomalacia presents clinical pictures that include decreased serum levels of calcium, phosphate and 25-hydroxyvitamin D and increased levels of alkaline phosphatase and PTH.

As previously mentioned, the active form of vitamin D, 1,25-dihydroxyvitamin D, acts at the level of vitamin D receptors in the intestine promoting its absorption. Therefore, any alteration in the route of metabolization of this vitamin significantly affects calcium absorption, its serum levels and vitamin D itself. In addition, as seen in other pathologies already mentioned, conditions such as poor nutrition, poor sun exposure, changes in gastric absorption, renal or hepatic failure may incite a decrease in vitamin D levels and thus lead to the development of osteomalacia.

In addition to low vitamin D levels, osteomalacia can also occur due to alterations in the metabolism of this vitamin, hypophosphatemia or by inhibition of bone mineralisation (Table 3-5).

Table 3-5. Causes of osteomalacia.

Vitamin D deficiency:	Inadequate nutrition
	Insufficient sunlight exposure
	Intestinal malabsorption
Abnormal metabolism of vitamin D:	Kidney failure
	Liver failure
	Medication
Hypophosphatemia:	Inadequate nutrition
	Excessive loss of phosphate through the kidneys
Inhibition of bone mineralization: Induced by:	Bisphosphonates
	Aluminium
	Fluoride

In osteomalacia PTH is also increased. This hormone is secreted by the thyroid in response to low levels of calcium in the blood. When serum levels of this mineral decrease, PTH is secreted to increase the activity of osteoclasts and consequently boost bone resorption. This hormone also acts at the level of the renal tubules, also promoting calcium resorption (Fig. 3-2).

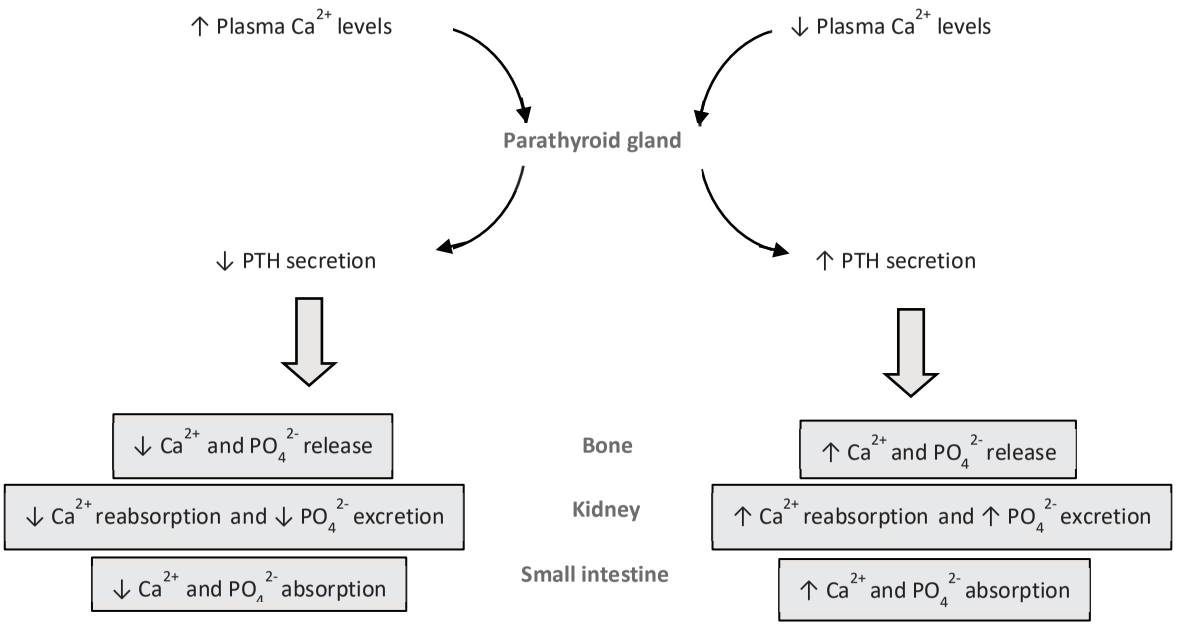

Fig. 3-2. The effect of calcium in the secretion and action of PTH.

With PTH hypersecretion, there is a marked increase in bone remodelling resulting in an irreversible loss of bone mass that significantly increases the risk of fracture.

3.1.6. Paget’s disease

Paget's disease is one of the bone diseases that affects the most people worldwide, being the second most prevalent after osteoporosis. This condition mainly affects the elderly and is more common in men. The aetiology of this disease is still not well known, but it is characterised by an accelerated and uncontrolled bone resorption followed by a disorganised deposition, leading to bone deformities and weakness. This bone disorganisation is due to an increase in the number and size of osteoclasts. The bones most frequently affected are the femur, the vertebrae, and the pelvis. This pathology can be classified as monostotic, affecting only one bone, or polyostotic, extending to two or more bones.

Although the aetiology of this pathology is still not completely known, several factors have been pointed out. The most recent studies indicate that Paget's disease results from a viral infection of the osteoclasts, but genetic factors may also be preponderant in the development of the pathology, heredity being one of the greatest risk factors. Besides genetic factors, environmental factors have also revealed an important role in the development of the disease. Factors such as eating a diet low in calcium and vitamin D and exercising with an excessive load on the bones can be considered risk factors for the development of the pathology. Viral infections of osteoclasts are caused by paramyxoviruses, namely measles virus, respiratory syncytial virus, and canine distemper virus.

The main symptom of this pathology is bone and joint pain, resulting from bone deformations which can lead to bowing of the limbs and fractures, due to their fragility. Other frequent symptoms are hypercalcaemia, hearing loss, tinnitus, and headache due to alterations in the temporal bone or skull, respectively. Besides bone and joint complications there are also neurological and cardiovascular alterations such as paresis, paraesthesia, heart failure and increased cardiac output.

Biochemical markers in Paget’s disease diagnosis

The diagnosis of this pathology often occurs in a late stage of the disease, since in many individuals this pathology is asymptomatic. The diagnosis is usually made by X-ray or bone scintigraphy.

Paget's disease is characterized by a high rate of bone remodelling. Thus, it is expected that biochemical markers of bone renewal provide important information for the diagnosis of this pathology and are even more relevant in the monitoring and response to treatment.

The most used biochemical marker in the diagnosis of this disease is alkaline phosphatase, a biochemical marker of bone formation, which is increased in about 85% of patients. Normal alkaline phosphatase values may be indicative of monostotic disease or of a primary stage of this pathology. The evolution of the disease, considering the variations in alkaline phosphatase, tend to increase slowly and the progression of this pathology can last for decades.

Total alkaline phosphatase reflects not only the activity of the bone, but also liver function. Thus, if other liver enzymes are increased, it is important to consider other biochemical markers since alkaline phosphatase values may be altered by changes in liver function.

More recent literature has indicated the use of new biochemical markers of bone formation, such as type I pro-collagen peptides (PINP and PICP), osteocalcin and specific alkaline phosphatase, and biochemical markers of bone resorption, such as collagen catabolism products and TRAP.

Collagen catabolism products, namely PID and DPD, provide important indications of disease progression following treatment with bisphosphonates. PID and DPD are eliminated in free and conjugated forms in the urine and there is a marked decrease in the latter because of treatment. Therefore, these are the biochemical markers used for monitoring the treatment of Paget's disease with bisphosphonates.

CTX also provides us with important information for monitoring the evolution of the disease. The non-isomerised form of this molecule (α-CTX) spontaneously converts to β-CTX as the bone ages. What occurs in Paget's disease is an increase in the non-isomerised form to the detriment of the isomerised form due to constant bone remodelling. With therapy, there is a greater decrease in α-CTX compared to β-CTX.

3.2. Joint diseases

The joint is fundamental for locomotion and its function is complex. The tissues present in the joint allow coordinated and frictionless movement. Cartilage allows the movement associated with the joint, is aneural and

avascular and derives essentially from the synovial fluid. The tissue is composed of metabolically active chondrocytes, proteoglycans and collagen and the extracellular matrix is essentially composed of water. The collagen fibres are arranged in different orientations and bring together proteoglycans and water molecules. The chondrocytes that make up cartilage are central to joint maintenance and repair.

Subchondral bone also has two important functions for the joint. In addition to providing nutrients for the cartilage it also lessens the impact of movement, so the joint is not overloaded. Ligaments provide stability to the joint and prevent the development of abnormal movements. The entire joint is surrounded by a fibrous capsule where, inside, resides the synovium. The synovium is highly innervated and vascularised and secretes a viscous fluid (synovial fluid) that lubricates the joint.

The synovial fluid is a viscous and mucinous substance, consistency conferred by hyaluronic acid and lubricrin, produced by chondrocytes and synovial cells. The composition of this fluid is like plasma and together with bones, muscles, ligaments and joints it forms the Locomotor System. Cartilage provides a flat surface with an exceptionally low coefficient of friction, allowing effective gliding during joint movement without causing any damage. In the cartilage of a healthy adult at rest, the chondrocytes are inactive cells with low proliferation. These chondrocytes have receptors for different components that are activated by mechanical stimulation releasing matrix-degrading enzymes and cytokines. The matrix degrading enzymes found in the joint include aggrecanases and collagenases which constitute some metalloproteinases.

Joint diseases can affect cartilage, subchondral bone, and synovium, putting joint function at risk. Joint diseases can include degenerative, inflammatory, autoimmune, or infectious diseases, including rheumatoid arthritis, systemic lupus erythematosus, spondylarthritis, osteoarthritis, osteonecrosis, gout, and septic arthritis. These diseases affect a considerable percentage of the population, with osteoarthritis affecting the largest number of individuals. These diseases significantly diminish the quality of life of sufferers and can involve a great social burden.

The diagnosis of these pathologies can be made based on biochemical changes in the synovial fluid. Macroscopic analysis, cell count, Gram staining and crystal analysis are tests carried out in the diagnosis of joint diseases that allow the identification of diseases of inflammatory origin or not and of infectious origin, such as septic arthritis.

3.2.1. Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease that affects about 1% of the population, with women three times more likely to develop it than men, especially women between the ages of forty and sixty.

This autoimmune disease manifests itself systemically and is related to joint inflammation, which often becomes chronic. It is known that this process is related to the production of antibodies by the immune system that triggers a series of events that culminate in joint destruction and erosion. With the decrease in physical function comes a decline in quality of life with a strong impact on the patient's social life due to the high risk of complications. Rheumatoid arthritis is associated with a higher risk of morbidity and premature death. Besides the articular manifestations, this pathology affects other organs. Extra-articular manifestations include vasculitis and pulmonary problems.

The factors responsible for the development of this pathology remain to be understood, but it is known that it is a multifactorial disease, therefore resulting from the combination of environmental factors and genetic predisposition.

The current understanding of the genetic component associated with the development of rheumatoid arthritis is still not well defined. Several studies using epigenetics have been carried out and have identified several genes that predispose to the development of this pathology. Most of these genes are involved in mechanisms that confer immunity or other inflammatory diseases. Dysfunctions of leucocytes and fibroblasts have been associated with the development of rheumatoid arthritis.

In addition to genetic factors, several environmental factors have been described in relation to the development of this pathology. Illiteracy, low socioeconomic status, and smoking are the most important. Some microorganisms have also been associated with the development of this pathology, but it has not yet been possible to prove the relationship between them.

Symptoms of rheumatoid arthritis include non-specific signs such as joint pain, tenderness and swelling. These symptoms may progress slowly. Symmetric polyarthritis, morning stiffness, involvement of the metacarpophalangeal or metatarsophalangeal joints are specific signs of the disease. These symptoms are related to joint inflammation that is triggered

by the presence of cytokines that interact with white blood cells and trigger an inflammatory reaction.

Synovial fluid has a composition like that of plasma, containing cells such as macrophages, fibroblasts, lymphocytes, mast cells, dendritic cells, B cells and T cells. These cells have a complex and interrelated function that is decisive in the development and pathogenesis of rheumatoid arthritis.

Macrophages and monocytes are responsible for producing pro-inflammatory mediators, namely several cytokines such as tumour necrosis factor (TNF), interleukins (IL-1, IL-6, IL-10, IL-12, IL18, IL-15) granulocyte and macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and transforming growth factor beta (TGFβ). The production of these mediators stimulates the activation and expression of chondrocytes and metalloproteinases leading to cartilage degradation. It is considered that macrophages and monocytes are the centre of the inflammation causing this pathology. Besides producing cytokines, these cells also act as antigens stimulating the T-cell response, intensifying joint destruction.

Biochemical markers in rheumatoid arthritis diagnosis

Early diagnosis and intensive treatment are essential to control the disease and ensure a better quality of life, avoiding reaching the disability that rheumatoid arthritis can confer. When the diagnosis is made at an early stage of the disease it is possible to prevent the destruction of the joint and the bone.

With the development of this autoimmune pathology, changes in cartilage and bone metabolism occur. Cartilage turnover occurs in a controlled way, with a balance between its degradation and formation. With the installation of the inflammatory process, there is an imbalance between the formation and degradation of cartilage, with an increase in its destruction. This process can be monitored by taking into consideration the molecules that are produced. Cartilage is predominantly composed of type II collagen and proteoglycans.

The main mediators of cartilage degradation are proteolytic enzymes, metalloproteinases (MMPs), and aggrecanases, more specifically disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS).

Proteoglycans are degraded by both enzymes, while type II collagen is only degraded by MMPs. With the onset of the inflammatory process there is a

significant increase in cartilage degradation and with it the release of peptide fragments of collagen and proteoglycans.

The collagen degradation product CTX-II is obtained by the action of MMPs, and its monitoring has proved useful in controlling cartilage degradation.

Despite developments in biochemical marker studies, the diagnosis of this pathology is made following other guidelines. For the diagnosis of rheumatoid arthritis some tests considered subjective are performed, such as pain assessment, duration of morning stiffness, level of tiredness and physical limitations. These parameters are quantified using visual or numerical scales. Despite being considered a subjective evaluation, this measurement becomes important in the course and evolution of the disease, allowing an estimation of the response to treatment. According to the guidelines, it is necessary to perform physical and laboratory tests (Table 3-6), as well as hand and foot X-rays, which may be normal at the beginning of the disease but become important for monitoring the pathology.

Table 3-6. Physical exams and laboratory tests used in the diagnosis of rheumatoid arthritis.

Physical exams	Laboratory tests
Swollen joint count	Sedimentation rate
Loss of movement assessment	C-reactive protein
Evaluation of crepitus	Hemogram
Assessment of joint instability and misalignment	Rheumatoid factor
Evaluation of deformed joints	Electrolytes levels
Extra-articular manifestations	Creatinine level
	Liver enzymes levels
	Synovial fluid analysis

In rheumatoid arthritis, the blood sedimentation rate and C-reactive protein content are usually increased. The hemogram of patients in active disease shows an increase in the number of platelets (thrombocytosis) and normocytic normochromic anaemia. Rheumatoid factor dosage must always be requested when there is a suspicion of rheumatoid arthritis and consists of an investigation of the antibody produced in this autoimmune disease. Despite being specific for this pathology, it is only positive in around 70% of patients and can still be positive in 5% of the normal population.

3.2.2. Gout

Gout is usually associated with increased serum uric acid level (hyperuricemia) and consequent crystallisation of uric acid in the synovial joint fluid, a consequence of abnormal purine metabolism. This pathology affects one in every two hundred individuals and is more prevalent in men (it affects four times more men than women).

Gout is one of the pathologies which affects the joints and triggers inflammatory processes associated with pain and consequent impairment of quality of life. Its incidence increases with advancing age and the number of gout cases has increased considerably in recent years. Poor nutrition, a sedentary lifestyle, increased average life expectancy and the increased prevalence of obesity may explain the expansion of the disease.

In this pathology it is possible to consider two clinical phases. In the first phase, the individuals present acute, intermittent, and self-limited episodes, which usually last for seven or ten days. If during this phase hyperuricaemia is not treated, or is inadequately treated, it may transition to the second phase in which the condition manifests itself chronically with polyarticular episodes and recurrent crises.

Besides the morbidity attributed to the disease itself, this joint pathology is related to other conditions that are highly detrimental to the individual's quality of life, such as insulin resistance syndrome, hypertension, congestive heart failure, nephropathy and disorders associated with increased cell renewal. With the deposition of crystals in the synovial fluid, clinical manifestations occur, such as pain, inflammation, and joint damage, affecting peripheral joints, more specifically the metatarsophalangeal joint.

Hyperuricemia is considered the most important risk factor for the development of this pathology. Uric acid values equal to or greater than 7.0 mg/dL are considered high values. Individuals with these values are at serious risk of developing gout.

The main cause of hyperuricaemia is the deficient excretion of uric acid and these variations in renal excretion are related to genetic factors. Changes in the SLC22A12 gene, which encodes a uric acid transporter, can lead to an increase in its reabsorption, causing the development of hyperuricaemia.

Renal failure is also a major risk factor, and individuals with kidney problems require greater attention. The incidence of this pathology in

patients undergoing haemodialysis is quite high, affecting about 5% of these individuals during the first year of treatment alone.

Dietary factors have been widely studied and their relationship to the development of gout has long been known. Diets rich in meat and seafood are predominant in the development of this pathology. In contrast, the consumption of low-fat dairy products seems to have a protective action.

Besides a diet rich in purines, excessive consumption of alcoholic and sugary drinks also contributes to the development of hyperuricaemia. Not all types of alcoholic drinks contribute equally to the development of this pathology. The risk of developing gout is greater with the consumption of beer when compared to wine, which does not carry a great risk. This is essentially due to its purine content.

Purine metabolism

Hyperuricaemia may be due to excessive uric acid production from the degradation of ingested purines. The purine degradation pathway (Fig. 3-3) begins by converting adenosine monophosphate (AMP) to inosine and then to hypoxanthine. Guanosine monophosphate (GMP) gives rise to guanine and then xanthine. Hypoxanthine is converted to xanthine by the enzyme xanthine oxidase which in turn converts xanthine to uric acid. Uric acid is easily converted to allantoin which is a soluble and easily excreted compound.

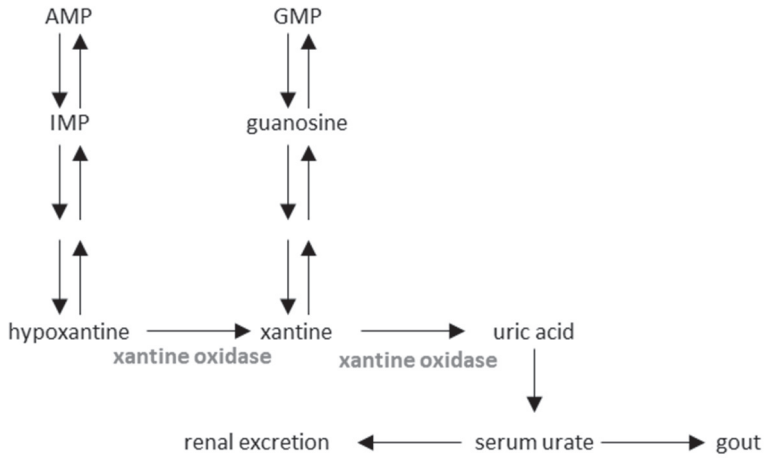

Fig. 3-3. Purine degradation pathway.

However, obtaining purines is not exclusive to the diet. There are also endogenous mechanisms that lead to its production.

The biosynthesis of purines (Fig. 3-4) occurs with the formation of AMP and GMP, which are part of the structure of nucleic acids (deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)). This pathway is regulated by negative feedback. When AMP and GMP products reach high concentrations, negative feedback is provided, that ceases the activity of phosphoribosyl pyrophosphate (PRPP) synthetase and thus balances nucleotide levels.

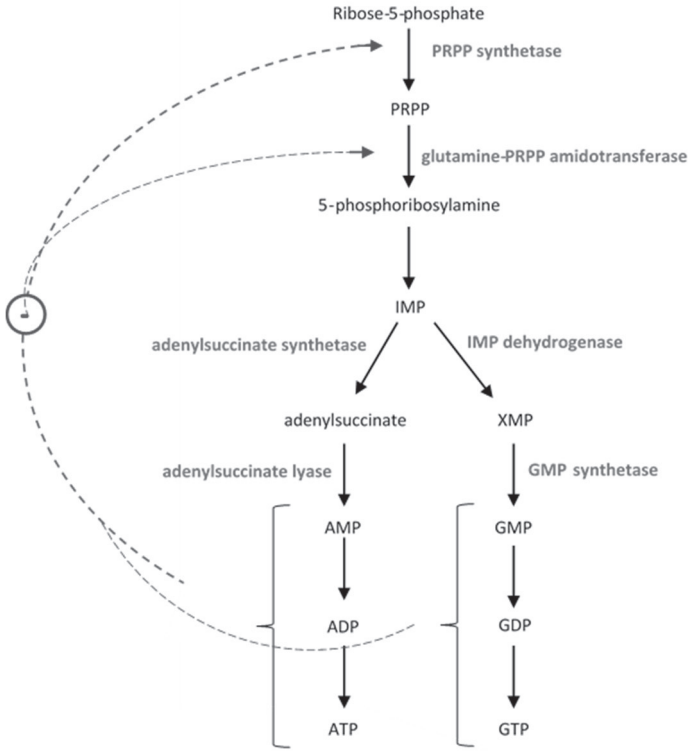

Fig. 3-4. Endogenous production of purines.

When plasma uric acid values exceed 7.0 mg/dL, and gathering other factors such as pH, temperature and binding to proteins, this salt crystallises, especially in the synovial fluid of the joints, triggering the inflammatory process.

With the deposition of uric acid salts in the joint there is an activation of monocytes and macrophages, which capture them by phagocytosis. After being phagocytosed, these crystals activate the NLRP3 protein complex by still unknown mechanisms, leading to the release of IL-1β from the cell. Consequently, endothelial activation occurs and thus leukocyte recruitment. With the release of inflammatory mediators by the leukocytes, the inflammatory process characteristic of this pathology is installed.

Biochemical markers in gout diagnosis

The diagnosis of this clinical condition is made by considering some of its typical symptoms. Acute and severe pain with greater incidence in the metatarsophalangeal joint are obvious and alarming signs for the diagnosis of gout. During these acute manifestations, the circulating uric acid values may be normal, which does not exclude the diagnosis of this pathology. In addition to blood uric acid values, it is also important to determine C-reactive protein values, which are expected to be high during these episodes.

International recommendations elucidate the importance of synovial fluid aspiration for diagnosis. The search for crystals in the synovial fluid of inflamed joints is the main means of assessing the presence or absence of this pathology. It can also be beneficial for diagnosis in asymptomatic periods. In addition to the crystal search, it is important to perform a bacteriological examination with the elaboration of a Gram stain to exclude the possibility of septic arthritis. Imaging can also help as it allows bone and joint damage to be assessed.

According to the Portuguese Society of Rheumatology, after the diagnosis of gout it is necessary to screen for some chronic conditions such as diabetes mellitus, hypertension, dyslipidaemias, and coronary heart disease, as there is a strong relationship between the presence of gout and these clinical conditions. In addition, it is important to check kidney function as there is an increased risk of death due to chronic kidney disease in patients with gout.

3.2.3. Osteoarthritis

Degenerative joint diseases are related to the degeneration of cartilage, menisci, and inflammation of the subchondral membrane, eventually affecting all joints. Joint degeneration affects the individual's physical condition. The cartilage is worn away, promoting friction between bones, and causing pain, trauma, and ligament rupture. Degenerative joint diseases are essentially related to the ageing process.

Osteoarthritis is a degenerative joint condition and is one of the main causes of chronic pain and mobility deficit. As it is a disease that sets in progressively, it may remain silent for long periods of time. It affects the elderly and the female population the most, and it is estimated that over 250 million people in the world suffer from this comorbidity. Considering that this pathology has no cure, it is expected to be one of the main causes of disability and to have a considerable socioeconomic impact. Currently, the costs incurred by osteoarthritis represent around 1 to 2.5% of the gross national product in countries such as the United Kingdom, France, Australia, Canada, and the United States of America.

Besides being related to the ageing process, this disease is also characterised by a lack of compensation in the repair process of damaged cartilage. As cartilage is avascular, and the supply of nutrients to the joint is ensured by the subchondral bone, there is a restriction in the supply of nutrients and oxygen. With the lack of these nutrients, the chondrocytes try to compensate by clustering in damaged areas, which increases the concentration of growth factors in the matrix. With this dysregulation and decrease in nutrients there is an imbalance that culminates in joint degradation.

As previously mentioned, chondrocytes have receptors for extracellular matrix components. Their activation stimulates the production of enzymes responsible for joint degradation such as metalloproteinases, aggrecanases and collagenases.

In osteoarthritis, early joint degradation is due to MMP-3 and ADAMTS-5, which degrade the aggrecan. Consecutively, there is increased activity of the collagenases, in particular MMP-13, which is highly efficient in degrading type II collagen.

This whole process, (1) increased synthesis of proteinases, (2) apoptosis of chondrocytes and (3) inadequate synthesis of cellular matrix components, culminates in the formation of a matrix unable to support movement,

leading to joint collapse. As it is not innervated, no clinical signs are produced, leading to a late diagnosis.

The inflammation that arises from this process, characteristic of osteoarthritis, is related to the cartilage debris resulting from its degradation. This debris enters the synovial cavity and is detected by macrophages which respond by producing pro-inflammatory mediators. With the inflammatory process installed, there is an increased production of proteinases.

The pathogenesis of osteoarthritis can be divided into three phases (Fig. 3-5), culminating in the progressive alteration of the morphology, composition, and properties of the joint, and in the imbalance between catabolic and anabolic events of the chondrocytes.

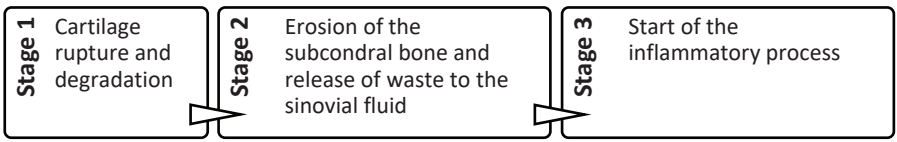

Fig. 3-5. Pathogenesis of osteoarthritis.

Another primary risk factor for the development of osteoarthritis is overweight and obesity. With weight gain there are several changes such as altered gait and joint biomechanics with slower gait and shorter and wider steps and longer support duration. These changes in gait trigger changes in the cartilage support regions that are involved in the evolution of osteoarthritis. In addition, obesity triggers a chronic low-grade inflammatory state. According to the literature, pro-inflammatory cytokines released by adipose tissue (IL-1, IL-6, TNF, leptin, and adiponectin) have a great influence on cartilage.

Biochemical markers in osteoarthritis diagnosis

It is common knowledge that osteoarthritis affects all structures of the joint through different processes. Biochemical markers have shown an important role in measuring the different pathological processes linked to the disease. Cytokines, enzymes, extracellular matrix constituents, collagen degradation products and proteoglycans can be used as biochemical markers. These markers can be detected at an early stage of the disease even before there are radiographic changes.

The literature identifies two potential categories of biomarkers that can be used in the diagnosis and evaluation of osteoarthritis. One category includes

the extracellular matrix constituents of the joint and the second category covers enzymes or cytokines that metabolise joint tissue molecules.

In the first category, the most important biochemical marker is pyridinoline which is abundant in cartilage and bone and is released during the resorption process. It is excreted intact in the urine and is a good indicator of joint destruction and metabolism. However, as it is present in considerable amounts in different tissues of the joint, high values can result from various factors such as subchondral bone sclerosis, synovial degeneration, or even joint degeneration.

MMPs are proteolytic enzymes that belong to the second group of biomarkers as well as aggrecanases and tissue inhibitors of metalloproteinases, pro-inflammatory cytokines (IL-1, IL-6, TNF-α), prostaglandin E2 (PGE2), growth factors and C-reactive protein.

Research into new biochemical markers has intensified in recent years as they represent an important discovery for monitoring osteoarthritis. The most widely used diagnostic and monitoring method is radiography. This has the advantage of being quick, inexpensive, easy to access and allows high resolution images to be obtained. However, excessive exposure to radiation is not advised and does not provide information about the synovial tissue.

In addition to the above-mentioned disadvantages, radiography only allows significant changes to be visualised after a period of one or two years. In contrast, biochemical markers allow changes to be observed after a few months, and biomarkers are therefore preferable for disease monitoring.

3.3. Muscular diseases

Muscle is the most abundant tissue in the human body and is composed of connective tissue, blood vessels, nerves, and contractile material that in unison influence muscle function. The muscle fibre is the structural unit of the muscle. It is highly elongated and specialized. Several fibres arranged in parallel originate the muscle bundle, which is surrounded by the perimysium.

The perimysium is composed of well-organized collagen fibres that accompany the muscle fibres. These oriented longitudinally form dense bands that are interposed by transversal collagen fibres forming a three-dimensional network that reacts to external deformations.

Each muscle fibre is surrounded by the endomysium that is formed by a highly organised network and serves to support the muscle fibres. The epimysium is essentially made up of collagen. Its rigidity increases with age and is thought to be involved in the transition of strength between muscles.

These three layers are essentially composed of collagen and other connective tissue proteins, such as elastic fibres and proteoglycans. The extracellular matrix of the muscle is a highly organised network that maintains the balance between deposition, remodelling and degradation of the muscle in response to any external aggression.

The size of the muscle and muscle fibre changes according to certain physiological and pathological conditions. An increase in muscle fibre size is seen in response to mechanical overload or hormonal stimulation with testosterone. On the contrary, muscle loss can be identified because of ageing, periods of food shortage, cancer, diabetes, rest, loss of nerve stimulation or the use of corticoids.

Muscle has a regenerative capacity due to its composition of multinucleated cells. The regeneration of this tissue depends on the fusion of unicellular cells, also called satellite cells. The mechanism of muscle regeneration has long been studied and is of great interest to the scientific community because it helps to develop new cellular therapies.

After muscle damage, there is a proliferation of satellite cells. These differentiate into myoblasts and then originate the multinucleated myotubes. There is great interest in studying this mechanism of regeneration as it may allow the development of cellular therapies where muscle degeneration occurs, as in the case of muscular dystrophies.

Apart from ageing, other conditions may induce these changes in skeletal muscle. The main biochemical changes in muscle are related to collagen. Mutations in the genes responsible for collagen type expression can induce myopathies. With ageing there is also a decrease in collagen type I and an increase in type III. These variations can induce muscle deterioration.

The extracellular matrix of the skeletal muscle is elemental in the transmission of force and response of the muscle. This muscle changes with age, contributing to the deterioration of muscle mechanical properties observed with age and to the development of certain pathologies.

3.3.1. Muscular dystrophies

Muscular dystrophies are hereditary disorders resulting from the involvement of one or more genes that define muscle function. The most common muscular dystrophies are facioscapulohumeral muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, Emery-Dreifuss dystrophy, myotonic dystrophy, waist muscular dystrophy and congenital dystrophies (Table 3-7).

Of all the mentioned muscular dystrophies, Duchenne muscular dystrophy is the most severe form of these hereditary disorders, however all have varying degrees of muscle loss. This is the most common muscular dystrophy in childhood and primarily affects males. Becker muscular dystrophy is considered a variation of the former and manifests itself in a smaller number of individuals.

Epidemiological data on the remaining muscular dystrophies indicate that myotonic dystrophy is the most prevalent in adults, followed by facioscapulohumeral muscular dystrophy. Congenital muscular dystrophies show variations in prevalence according to geographical area. Ulrich congenital muscular dystrophy was originally reported as the most common subtype of this group, although Fukuyama muscular dystrophy is the most common type in Japan.

The muscles affected by muscle weakness are those of the lower and upper limbs, the axial and facial muscle. These can be affected to different degrees, with severity. In more specific muscular dystrophies, the muscle involvement may be different, affecting the respiratory muscles, smooth muscles, heart muscle or muscles involved in swallowing. Other organs and tissues may also be affected, notably the brain, inner ear, eyes, or skin.

In all types of muscular dystrophies, it is possible to detect the presence of muscle weakness, which can be differentiated according to its degree and nature. Respiratory impairment is also common to the various types of dystrophies, although it varies according to the degree of the disease. This impairment is normally associated to the loss of the ability to walk. The weakening of the muscles involved in coughing and swallowing results in compromised airway clearance, imposing the need for lung recruitment manoeuvres and assisted coughing.

Table 3-7. Summary of muscular dystrophies.

Muscular Dystrophy	Heritage	Gene	Protein	Clinical conditions
Muscular facioscapulohumeral dystrophy	Autosomal dominant	*DUX4*	Reorganization of sub telomeric chromatin	Muscular weakness in facial muscles; rare commitment of cardiac and respiratory function.
Muscular Duchenne dystrophy	Linked to the X chromosome	*DMD*	Dystrophin	General muscular weakness; cognitive changes; commitment of cardiac and respiratory function.
Muscular Becker dystrophy	Linked to the X chromosome	*DMD*	Dystrophin	Clinical condition like Duchenne muscular dystrophy
Emery-Dreifuss dystrophy	Variable: Autosomal dominant Autosomal recessive Linked to the X chromosome	*LMNA*	Emerin	Premature development of muscular contractures; loss of muscular mass in upper and lower limbs and in abdominal muscles; development of cardiomyopathy; commitment of respiratory function in adults;
Myotonic dystrophy	Autosomal dominant	Variable: *DMPK* *CNBP*	Protein kinase	Slow reasoning; somnolence; fatigue; abnormal behaviour; myotonia; cataracts; insulin resistance

Belt muscular dystrophy	Variable: Autosomal dominant Autosomal recessive	Variable	Several: • Sarcoglycan • Dystroglycan • Telethonin • Titin	Muscular weakness in belt muscles; changes in intellectual abilities.
Congenital dystrophy	Autosomal recessive	*LAMA2*	Laminin α2	Premature development; hypotonia; muscular weakness; myopathic changes; contractures.

Muscular dystrophies diagnosis

Muscular dystrophies include a large group of pathologies that are based on the same symptoms such as muscle weakness, loss of skeletal muscle and bone mass. They all have a hereditary character and the difference between them is solely related to the genetic alteration involved.

The diagnosis of muscular dystrophies is essentially based on the identification of the genetic defect. A correct and early diagnosis is essential to increase the quality and life expectancy of these patients, as a cure is still not possible. The doctor must perform a physical examination, determine the extent of the symptoms, and evaluate the family history.

The search for the genetic mutation is performed through gene sequencing which must be preceded by the performance of other tests such as the determination of the serum creatine kinase level, to evaluate muscle damage, electromyography, and magnetic resonance imaging to determine muscle involvement. A muscle biopsy should also be performed, which is fundamental in the evaluation of these patients.

3.3.2. Metabolic myopathies

Metabolic myopathies are related to the inability to maintain adequate adenosine triphosphate (ATP) production in muscle. They are inherited primary muscle disorders related to fatty acid oxidation, glycogen storage or mobilisation, glycolysis, the Krebs cycle, or the respiratory chain.

The main sources of energy used by the muscle for ATP production are glycogen, glucose, and fatty acids. Glucose is metabolised by glycolysis in the cytoplasm, producing pyruvate which enters the mitochondria. On the other hand, short and medium chain fatty acids can pass freely through the mitochondria. Once inside, these substrates are converted to acetyl-coenzyme A (acetyl-CoA) which participates in the Krebs cycle (Fig. 3-6) to produce ATP.

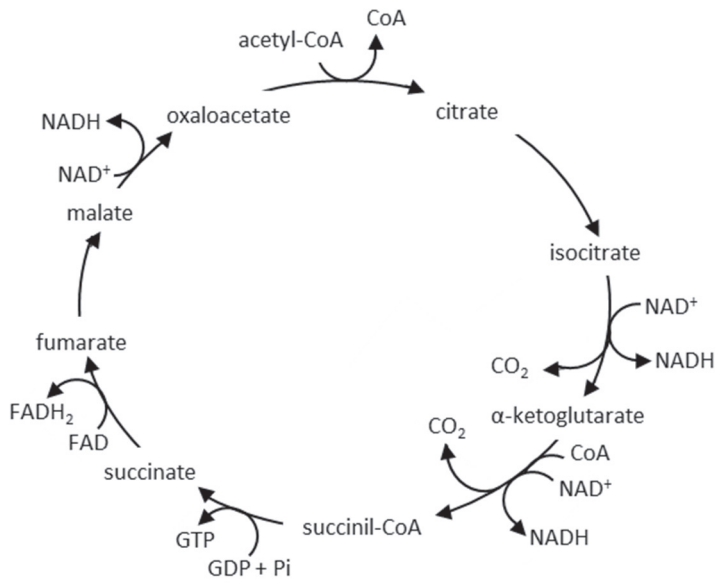

Fig. 3-6. The Krebs cycle.

Disturbances in carbohydrates metabolism

This group of pathologies includes disorders affecting the synthesis and/or degradation of glycogen, a polysaccharide produced in the liver and muscles for storage of glucose. This polysaccharide is degraded whenever necessary to obtain glucose.

Myophosphorylase deficiency, better known as Mcardle's disease, is the best-known disorder of carbohydrate metabolism and the most common genetic myopathy. These patients usually show muscle weakness, myalgias, cramps and exercise intolerance. They also have high creatine kinase levels.

Glycogenolysis is the metabolic pathway that allows glucose to be obtained through the degradation of glycogen. Mutations on chromosome 11 compromise the function of myophosphorylase, which is responsible for converting glycogen into glucose-1-phosphate, which cannot be converted by the enzyme phosphoglucomutase into glucose-6-phosphate. Consequently, there is no glucose formation in the muscle, which is necessary to produce ATP.

Glycogenolysis and glycolysis are aimed at energy production during short-term exercise. Defects in glycogen or glucose metabolism tend to develop symptoms like those of Mcardle's disease. This disorder in carbohydrate metabolism is considered a rare myopathy that leads to the accumulation of glycogen in the muscle. As previously mentioned, this pathology is of genetic cause, with autosomal recessive inheritance, and occurs due to a lack of the enzyme myophosphorylase, resulting from a mutation in the PYGM gene present in chromosome 11 (q13.1).

The clinical manifestations of this disease are like those seen in other carbohydrate metabolism disorders, and include constant fatigue, exercise intolerance, rhabdomyolysis and myoglobinuria, which may be accompanied by renal failure. It develops mainly during adolescence and the diagnosis must be confirmed by muscle biopsy.

Disturbances in lipid metabolism

At rest and during long periods of low intensity exercise, the main source of energy used by the muscle is fatty acids. Short and medium chain fatty acids pass freely through the mitochondria whereas long chain fatty acids need to combine with carnitine to be transported. This transport is mediated by carnitine palmitoyl transferase (CPT) I and II. In the mitochondrial matrix, fatty acids undergo cleavage by β-oxidation enzymes and give rise to 2-carbon fragments (acetyl-CoA) in each cycle.

Often what is found in this type of disorder and what justifies the inefficient transport of fatty acids is a lack of carnitine or CPT I or II deficiency, or even defects in mitochondrial β-oxidation. These disorders are of genetic origin with autosomal recessive inheritance. Symptoms may develop throughout life, but the most severe and conditioning forms manifest early in childhood. The most common symptoms are myalgias, stiffness, muscle weakness and myoglobinuria. Patients usually present with acute attacks interspersed with asymptomatic periods. These attacks are often induced by various factors such as exercising for long periods, high fat intake, exposure to cold, fever, stress, and some medications, notably diazepam and ibuprofen.

One of the most common lipid metabolism disorders is late type II glutaric aciduria. This disorder is caused by a deficiency in the β-oxidation enzyme acyl-CoA dehydrogenase, essentially resulting from deficiency of the α or β subunit of this enzyme. Glutaric aciduria type II presents as the form with neonatal development, but without congenital abnormalities in the foetus. The most common symptoms of this disorder include vomiting, lethargy,

hypoglycaemia, metabolic acidosis, and oxidative stress. Muscle involvement is obvious and includes muscle pain and weakness.

Conclusion

Biochemical markers are measurable biological parameters that allow, to a certain extent, to determine the progression of a disease or the response of a certain patient to a drug. With the installed disease there are several biochemical alterations that can be observed, common to bone, joint and muscle diseases. It is certain that these alterations are not the same, nor are they common to all individuals. But, in general, it is possible to measure and evaluate these alterations.

Currently, the use of biochemical markers is extended to various areas of medicine. In the diagnosis of bone diseases, biochemical markers of bone formation and resorption are well known. Their limitations have been determined, and so far, they are an integral part of diagnosis and treatment monitoring.

In joint and muscle diseases, the biochemical alterations verified do not allow for as much sensitivity and specificity when compared to the previous ones. Many of the biochemical markers used in diagnosis may be altered because of various factors, not always pathological.

This chapter recognises the interest in studying and validating the different biochemical markers of each musculoskeletal disease. Developments in this field have been promising and move towards increasing knowledge about the biochemical alterations of each disease. It is important to extend the research and determine new biochemical markers.

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Chapter 4

Biochemical Changes in Common Psychiatric Diseases

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List of abbreviations

5-HT: 5-Hidroxytriptamine (Serotonin)
Ach: Acetylcholine
BDNF: Brain Derived Neurotrophic Factor
CB1: Cannabinoid type 1 receptor
D: Dopamine
DSM-5: Diagnostic and Statistical Manual of Mental Disorders – 5th edition
FGA: First Generation Antipsychotics
GABA: Gamma Aminobutyric Acid
GAD: General Anxiety Disorder
H: Histamine
HPA: Hypothalamus-Pituitary-Adrenal
IFN: Interferon
IL: Interleukin
LSD: Lysergic acid diethylamide
MAO: Monoamine Oxidase
MAOI: Monoamine Oxidase Inhibitors
MDD: Major Depressive Disorder

mRNA: messenger Ribonucleic Acid
NDRI: Norepinephrine and Dopamine Reuptake Inhibitors
NE: Norepinephrine
NMDA: N-Methyl-D-Aspartate
NT: Neurotransmitters
PD: Phobia-related Disorders
PFC: Pre-Frontal Cortex
REM: Rapid Eye Movement
SAD: Social Anxiety Disorders
SGA: Second Generation Antipsychotics
SNRI: Serotonin and Norepinephrine Reuptake Inhibitors
SSRI: Selective Serotonin Reuptake Inhibitors
TCA: Tricyclic Antidepressants
TNF-α: Tumour necrosis factor α
TRPV1: Transient Receptor Potential Vanilloid Type 1
TRS: Treatment-resistant schizophrenia
WHO: World Health Organization

Introduction

The American Psychiatric Association defines a mental disorder as a syndrome characterized by clinically significant disruption in an individual's cognition, behaviour, or emotional regulation, due to dysfunctions in psychological, biological or developmental processes of mental functioning. Mental disorders are associated with significant impairment in several areas of life, such as social relationships and occupational activities.

Mental health conditions are increasing worldwide. A 13% rise in mental health conditions and substance use disorders in the last decade (up to 2017) has been reported by the WHO. Mental disorders affect all ages, with children and adolescents accounting for 20%. Suicide is the second cause of death among those between 15 and 29 years of age. Two of the most common mental health conditions, depression and anxiety, cost the global economy 1 trillion US dollars each year.

Mental diseases do not always fit completely within the boundaries of a single disorder. However, to better understand and to facilitate communication among health professionals, DSM-5 classifies mental disorders in several categories. Understanding the aetiology and pathophysiology of these disorders should allow clinical benefits in the prognosis and the treatment of patients. This chapter will focus on mental

disorders which are common nowadays, such as depression, anxiety and schizophrenia, by reviewing hard evidence of the aetiology and pathophysiology of these disorders relevant for clinical psychiatry.

4.1. Major depressive disorder

This section will focus on major depressive disorder (MDD) as it is one of the most prevalent and debilitating psychiatric disorders, affecting one out of every five people during their lifetime. Women have a higher risk of first onset (15%), twice the occurrence of men. In 2008, the WHO considered MDD as the third cause of disease burden worldwide and projected that it would rank first by 2030. It is characterised by the presence of depressed mood and/or anhedonia (loss of interest or pleasure) for at least two weeks, causing significant changes in an individual's social or occupational functioning. Besides emotional regulation, this disorder affects memory and other cognitive functions, motivation, motor functions, and sleep regulation, and it also triggers undesirable neurovegetative symptoms. Moreover, it increases the chance of developing chronic medical illnesses. Decreased adherence to medical treatment is a major issue contributing to this secondary disability. MDD is usually a recurrent illness since more than 50% of patients who recover from a first depressive episode will have a second in the following six months unless they are undergoing antidepressant treatment. For those who never receive treatment, as many as 15% will commit suicide.

There are currently no biomarkers for MDD diagnosis or treatment response. For this reason, MDD diagnosis and management are often challenging for clinicians, because of its numerous presentations, unpredictable development and variable response to treatment.

4.1.1. Pathophysiology and treatment – present and future

The pathophysiology of depression most likely results from complex interactions between biological mechanisms and the environment. Some possible pathophysiological mechanisms of depression include altered neurotransmission, neuroendocrine dysfunctions, reduced neuroplasticity and inflammation.

A major pathophysiological hypothesis for depression is the monoamine hypothesis, suggesting an alteration in the monoamine pathways, including serotonin (5-HT), norepinephrine (NE) and dopamine (D). This theory emerged in the 1950s following the observation that drugs which depleted

these neurotransmitters (NT) triggered depressive symptoms. Evidence for this theory also came from the fact that the levels of these NT or their metabolites were reduced in patients diagnosed with MDD and were increased with antidepressant therapies. Most antidepressant drugs act by blocking the NT transporters present on the surface of the pre-synaptic membrane, thus inhibiting the reuptake of NT, which accumulate in the synaptic cleft and become more available to activate post-synaptic neurons. Table 4-1 shows the most common antidepressants, their respective biochemical targets and mechanisms of action.

As a result of their non-selective protein binding profile, tricyclic antidepressants (TCA) have undesirable side effects, including cognitive impairments, dry mouth and increased heart rate. To improve side effect profiles, pharmaceutical industries focused on the development of more selective reuptake inhibitors, leading to several *selective serotonin reuptake inhibitors* (SSRI). These agents are better tolerated but may also have some side effects including gastrointestinal symptoms, headaches, sustained sexual dysfunction and diminished REM sleep.

NE is also involved in mood regulation, as evidenced by the fact that medications that inhibit NE reuptake, such as TCA, *serotonin and norepinephrine reuptake inhibitors* (SNRI), and *norepinephrine and dopamine reuptake inhibitors* (NDRI), and those that increase NE secretion, such as mirtazapine, are effective antidepressants. Moreover, several studies implicate altered dopaminergic transmission and the mesolimbic pathway in the pathophysiology of depression. In fact, some critical symptoms of depression, including anhedonia and decreased motivation, are related to altered biochemical pathways of the reward system. Antidepressant agents, such as bupropion, which increase dopamine levels in the brain also provide indirect evidence for the role of dopamine in mood regulation.

Table 4-1. Antidepressants drugs, biochemical targets and mechanisms of action.

Drug class	Types of drugs	Membrane cell protein target	Mechanism of action	Common adverse effects
TCA	Nortriptyline Amitriptyline Imipramine Clomipramine	NE transporter 5-HT transporter H receptors Ach receptors α2-adrenergic receptors	NE reuptake inhibitor 5-HT reuptake inhibitor Effects on various receptors (H, Ach, α2-adrenergic).	Anticholinergic effects (confusion, blurred vision, dry mouth, urinary retention, constipation); Antihistaminergic effects (drowsiness, weight gain); Cardiovascular effects (dizziness, postural hypotension, increase in QRS interval); Gastrointestinal effects (nausea, vomiting); Neurologic effects (tremor, headaches, seizures)
SNRI	Venlafaxine Duloxetine Milnacipran	NE transporter 5-HT transporter	NE reuptake inhibitor 5-HT reuptake inhibitor	Adrenergic effects (dry mouth, increase in blood pressure, palpitations, sweating); Gastrointestinal effects (diarrhoea or constipation, nausea, vomiting); Neurologic effects (tremor, headaches, insomnia); Sexual effects (erectile dysfunction, anorgasmia, retarded ejaculation).

NDRI	**Bupropion**	**NE transporter D transporter**	**NE reuptake inhibitor D reuptake inhibitor**	**Adrenergic effects (dry mouth, increase in blood pressure, palpitations, sweating, insomnia, agitation, tremors, blurred vision, constipation, seizures)**
SSRI	Fluoxetine Sertraline Citalopram Escitalopram Fluvoxamine Paroxetine	5-HT transporter	Selective 5-HT reuptake inhibitor	Low intensity anticholinergic and antihistaminergic effects (dry mouth, drowsiness); Gastrointestinal effects (diarrhoea or constipation, nausea, vomiting); Neurologic effects (tremor, headaches, insomnia); Sexual effects (erectile dysfunction, anorgasmia, retarded ejaculation)
Other antidepressants	Mirtazapine	5-HT_2 receptor 5-HT_3 receptor α_2 -adrenergic receptor H_1 receptor	α2-adrenergic antagonist 5-HT_2 antagonist	Drowsiness, weight gain, constipation, blurred vision
	Trazodone	5-HT receptor	5-HT partial antagonist	Sedation, priapism
	Agomelatine	Melatonin 1 & 2 receptors 5-HT_{2C} receptor	Melatonin 1 & 2 agonist 5-HT_{2C} antagonist	Headache, drowsiness, nausea

Key: 5-HT, Serotonin; Ach, Acetylcholine; D, Dopamine; H, Histamine; NE, Norepinephrine; SSRI, Selective Serotonin Reuptake Inhibitors; SNRI, Serotonin Norepinephrine Reuptake Inhibitors; TCA, Tricyclic Antidepressants.

Overall, the monoamine deficit theory is, however, unable to explain the 3 to 4 weeks latency interval necessary to observe therapeutical effects after beginning antidepressant treatments, since these drugs lead to a rapid increase of monoamine NT in the synaptic clefts. Authors suggest that pre- and post-synaptic NT receptors and post-synaptic intracellular signal transduction pathways may also be affected. The activation of post-synaptic receptors leads to a cascade of reactions involving protein G, cyclic adenosine monophosphate or kinase proteins, ultimately leading to the expression of genes, such as the *brain derived neurotrophic factor* (BDNF) which promotes neurogenesis and neuroplasticity. These properties allow the healthy brain to rapidly generate and eliminate synapses during adaptation and learning. Serum levels of BDNF have been shown to be reduced in patients diagnosed with MDD, suggesting a role of BDNF in the pathophysiology of depression. This theory has also been supported in animal experiments, namely by a knock-out experiment of BDNF in the hippocampus, which induced depressive behaviour in rats. Central or peripheral administration of BDNF has been shown to produce antidepressant-like effects, providing further support for the neurogenic hypothesis and the role of BDNF in the pathophysiology of depression. Fluoxetine has been shown to increase BDNF mRNA expression in the hippocampus, the ventral tegmental area and the nucleus *accumbens*, brain regions involved in memory and in the reward pathway. Although a BDNF deficit alone as an aetiological factor for depression appears to be an unlikely explanation, the BDNF–tyrosine kinase receptor B pathway, important for neuronal survival and for depressive neuropathology, has been suggested as a possible target for antidepressant medications.

Non-monoamine NT may also have a role in MDD pathophysiology. Accordingly, the drug agomelatine acts on melatonin receptors improving the regulation of circadian rhythms and minimizing depression symptoms (Table 4-1). Glutamate has also been implicated in mood regulation. Ketamine, a dissociative anaesthetic used for surgical procedures, has rapid antidepressant effects (within hours) and acts through the antagonism of N-methyl-D-aspartate (NMDA) receptors in GABAergic interneurons, reducing the inhibition of glutamate release in glutamatergic neurons. Besides increasing glutamate levels, ketamine also increases the production of BDNF in the hippocampus, suggesting that glutamate may be involved in mood regulation by promoting neuroplasticity. However, ketamine is a drug of abuse associated with cognitive impairments, schizotypal symptoms and neurotoxicity when high doses are used repeatedly. Research is in process to find safer agents and to determine a safe dosing treatment with ketamine,

in addition to molecules that could sustain the acute effects obtained with this drug.

Other pathophysiological mechanisms of depression have been discovered. These mechanisms include neuroendocrine impairments, such as in the hypothalamus-pituitary-adrenal (HPA) axis. It is well established that stress and depression are often related. In fact, stressful life events may precipitate depressive episodes in vulnerable individuals and childhood abuse or neglect increases the risk of depression later in life. Depression may be associated with a hyperactive HPA axis in response to lower levels of stress, leading to hypersecretion of corticotropin releasing factor from the hypothalamus, impaired negative feedback of the HPA axis, enlarged adrenal glands and hypercortisolism. This hypercortisolism has neurotoxic effects which may relate to depressive symptoms. Brain neuroimaging studies of depressed patients have shown a reduction in the metabolic activity of the pre-frontal cortex (PFC) and parts of the hippocampus, possibly related to the reduction of volume and neuronal atrophy in these regions. This volume reduction has been reported to be inversely correlated with the number of prior depressive episodes. Authors suggest a reduced inhibitory control of the atrophic PFC and hippocampus over the amygdala, a brain structure which regulates fear, anxiety and mood. Reduced volume of the PFC and the hippocampus could contribute to the impairments in executive function and memory often observed in patients with MDD.

Finally, recent evidence indicates a potential pathophysiological link between the immune system and neurobiological changes observed in MDD. In fact, research supports that antidepressant resistant MDD could be related to dysfunctional inflammatory and immune processes. Several studies have demonstrated that various inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumour necrosis factor-α (TNF- α), are overexpressed in the brain and periphery of patients with MDD. Moreover, chronic overstimulation of immune cells, such as microglia, monocytes and macrophages, significantly and adversely impacts neurobiological structure and function in MDD.

The ultimate goal in the development of new drugs in MDD would be to reverse the stress-induced cellular and molecular impairments, and hopefully the atrophy observed in brain regions of patients with MDD. The development of therapeutic agents, however, faces several hurdles, considering that MDD is a clinical heterogeneous entity. It is considered a syndrome widely believed to have a multifactorial aetiology and to have multiple subtypes, with environmental factors, such as stress and trauma,

interacting with a polygenic vulnerability. Further characterization of MDD and subtype specific pathophysiology, as well as development of biomarkers, will hopefully lead to treatments targeting selected abnormalities, more efficacious and with fewer side effects.

4.2. Anxiety disorders

Anxiety disorders are the most prevalent class of psychiatric conditions around the world (with a global prevalence of 7.3%) and are associated with significant comorbidity and morbidity. According to the WHO, about 264 million people globally suffer from anxiety disorders, representing an increase of 15% since 2005. Although anxiety is considered a normal reaction to stress and beneficial in some circumstances, anxiety disorders contrast with normal feelings of nervousness or anxiousness and encompass disproportionate long-lasting fear or anxiety which is difficult to control. Women are more likely than men to experience these disorders. Symptoms usually interfere with daily routines such as job performance, school performance and social interactions. Frequently, anxiety disorders precede the onset of other psychiatric disorders and may predict worse outcomes. They are often comorbid with depression, making the diagnosis and treatment very challenging.

There are several types of anxiety disorders, including generalized anxiety disorder (GAD), panic disorder, and various phobia-related (PD) disorders. Among these, social anxiety disorder (SAD) and specific phobia are the most common.

4.2.1. Pathophysiology and treatment – present and future

Anxiety disorders affect nearly 30% of adults at some point in their lives. Since their onset is at an early age, prompt and robust intervention by health professionals is a cost-efficient option. Despite the causes being generally unknown, it is likely that they involve a combination of genetic and environmental factors, as they usually run in families. It is estimated that genes contribute 30-50% to the development of these diseases. On the other hand, environmental factors such as stress and trauma probably contribute to the development of anxiety disorders through epigenetic mechanisms. Mothers who suffer from anxiety disorders and are not medicated exhibit altered DNA methylation of the glucocorticoid receptor gene (NR3C1) promoter region in cord blood and may increase the risk of an anxiety disorder in their children. Associations between anxiety disorders and

subsequent coronary heart disease seem particularly strong and emerging evidence points to associations of anxiety with stroke and diabetes.

The pathophysiology of anxiety is most likely a result of complex interactions among various mechanisms. The advent of neuroimaging studies, in the last two decades, has led to a better understanding of brain circuits involved in fear and anxiety. Excessive activity in limbic and paralimbic cortical areas has been regularly implicated in the pathophysiology of anxiety disorders. The parahippocampal gyrus and hippocampus are thought to play a key role in mediating fear and anxiety. Nonetheless, this understanding has not been translated into novel pharmacological treatments.

In fact, there has been a generalized paucity in the development of novel drug compounds to manage anxiety disorders. This might be due to the universal perception that currently available treatments are adequate. However, the literature indicates that only 60-85% of these patients respond to existing biological and psychological treatments and, among the responders, only half achieve recovery. Many explanations could account for the refractory nature of these disorders; nonetheless conventional treatments may not be effective for all patients and alternative pharmacotherapies should be developed.

Current treatments for anxiety disorders are described in Table 4-2. SSRI and SNRI are both first-line treatments for PD, GAD and SAD and have also been shown to be efficacious. According to a recent meta-analysis, escitalopram and duloxetine have the largest effect. Recommended duration of treatment varies from 3-6 months up to 1-2 years or even longer. These medications tend to be well-tolerated with short-lived side effects.

Table 4-2. Current treatments for anxiety disorders.

Drug class	Types of drugs	Membrane cell protein target	Mechanism of action
SSRI	Fluoxetine Sertraline Citalopram Escitalopram Paroxetine Paroxetine ER Fluvoxamine	5-HT transporter	Selective 5-HT reuptake inhibitor

SNRI	Duloxetine Venlafaxine	NE transporter 5-HT transporter	5-HT, NE (and D) reuptake inhibitor
TCA	Nortriptyline Amitriptyline Imipramine Clomipramine	NE transporter 5-HT transporter H receptors Ach receptors α2-adrenergic receptors	NE and 5-HT reuptake inhibitor Effects on various receptors (H, Ach, α2-adrenergic).
MAOI	Phenelzine	MAO	MAO inhibitor
Other antidepressants	Mirtazapine	5-HT_2 , 5-HT_3, α_2 adrenergic, H_1 receptors	5-HT_2, 5-HT_3, α_2, H_1 antagonist
GABAergic drugs	Pregabalin Gabapentin	voltage-dependent calcium channels	Unclear, may modulate calcium channels
Benzodiazepines	Clonazepam Alprazolam Lorazepam Oxazepam Chlordiazepoxide	GABA receptors	GABA-A agonist
Antipsychotics	Trifluoperazine Olanzapine Quetiapine	D_2, 5-HT_2, H_1 receptors	D_2 antagonist D_2, 5-HT_2 H_1 antagonist D_2, 5-HT_2 H_1 antagonist
Beta blockers	Propranolol	β-1, β-2 adrenergic receptors	β-1, β-2 antagonist
Antihistamines	Hydroxyzine	H_1 receptors	H_1 antagonist
Other anxiolytics	Buspirone	5-HT_{1A} receptors	5-HT_{1A} partial agonist

Key: 5-HT, Serotonin; D, Dopamine; ER, Extended Release; GABA, Gamma Aminobutyric Acid; H, Histamine; MAO, Monoamine Oxidase; MAOI, Monoamine Oxidase Inhibitors; NE, Norepinephrine; SSRI, Selective Serotonin Reuptake Inhibitors; SNRI, Serotonin Norepinephrine Reuptake Inhibitors; TCA, Tricyclic Antidepressants.

TCA were among the first classes of drugs used for anxiety disorders. They have a therapeutical effect by acting as reuptake inhibitors of serotonin and norepinephrine transporters. However, by blocking cholinergic (muscarinic M1), histamine H1 and alpha-adrenergic receptors, they also have several side effects, as mentioned in the previous section (Table 4-1).

Monoamine oxidase inhibitors (MAOI) are also older antidepressants only used as a third-line option due to important side effects and dietary restrictions. However, they may be considered in individuals suffering from SAD which were non-responsive to SSRI.

Mirtazapine is considered a mixed antidepressant for its broad pharmacological effect: presynaptic antagonism of the α-2 adrenergic receptor, postsynaptic blockade of 5-HT_2 and 5-HT_3 receptors, and antagonism of histamine-1 (H_1) receptors. It may have efficacy in improving anxiety but is normally prescribed as an adjunctive agent. Mirtazapine is generally safe for elderly patients since it presents fewer drug-drug interactions. Positive effects have been reported on sleep and appetite and they usually have fewer sexual side effects when compared to SSRI and SNRI.

Moreover, anticonvulsants like pregabalin and gabapentin have GABAergic properties. Despite limited research on the use of this class of drugs, there is strong evidence that the use of pregabalin in GAD has comparable effects to benzodiazepines and potential efficacy in SAD. Pregabalin acts on the alpha-2 delta subunit of calcium channels to reduce NT release. Gabapentin acts to modulate NT release on voltage-dependent calcium channels and has been prescribed off-label for anxiety.

Benzodiazepines are still among the most widely prescribed class of psychiatric drugs. They act as GABA-A agonists but are no longer used as first-line monotherapy for anxiety disorders. They can be used in the short-term on either a standing or as-needed basis for PD, GAD, and SAD in addition to SSRI or SNRI.

Antipsychotics, known for their D_2 receptor antagonist activity, have been used off-label for multiple conditions other than psychosis, including anxiety. There is a first-generation antipsychotic, a trifluoperazine, approved by the Food and Drug Administration (FDA) for the treatment of anxiety. Quetiapine, a second-generation antipsychotic (D_2, 5-HT_2 and H_1 antagonist), has been shown useful in monotherapy in GAD despite its poor tolerability. Olanzapine, also a D_2, 5-HT_2 and H_1 antagonist, is recommended in escalation strategies for GAD and PD. Nonetheless, further large-scale

research and longitudinal studies will be critical to establish the utility of antipsychotic drugs in anxiety disorders.

Propranolol, a beta-adrenergic antagonist, has been widely prescribed for SAD and performance anxiety. It is effective controlling autonomous reactions associated with these conditions, such as tremors, palpitations and diaphoresis. Since the main action of beta blockers is in the peripheral nervous system, little evidence is available to support its global use in anxiety disorders.

Although more effective agents are usually preferred to treat anxiety disorders, antihistamine drugs may also be considered. The use of H1 receptor antagonists in children/adolescents and pregnant women is considered safe. These drugs have sedative effects, are normally well tolerated but tend to develop tolerance over time. However, they should be avoided in older individuals with multiple comorbidities, for increasing the risk of cardiovascular diseases. Occasionally, children and older individuals may develop an undesired paradoxical behavioural effect with H1 receptor antagonists, characterized by motor and psychological agitation.

A different anxiolytic category, such as buspirone, known as a 5-HT_{1A} partial agonist, is commonly used as an adjunctive treatment with SSRI and SNRI, especially for GAD. However, it tends to be less effective than benzodiazepines and antidepressants. It also has a gradual onset of action, 10 days to 4 weeks, and non-negligible side effects, such as dizziness and headaches.

Over the last few years scientific research has aimed to develop new pharmacotherapies for anxiety disorders, shifting from the 5-HT, NE and GABA systems to biochemical pathways involving different NT, including glutamate and neuropeptides.

Glutamate is the primary excitatory neurotransmitter of the central nervous system, which binds to ionotropic receptors (NMDA; α-Amino-3-hydroxy-4-isoxazolepropionic acid – AMPA and metabotropic receptors – mGluR). Several preclinical studies have shown anxiolytic effects of mGluR modulators but so far none have had promising results. D-cycloserine is an NMDA partial agonist and is among the most widely studied glutamatergic agents in anxiety. However, large-scale studies have been discouraging in relation to its clinical benefits. In addition, memantine, an NMDA receptor antagonist approved for the treatment of Alzheimer disease, showed modest benefits in few patients suffering from GAD. Ketamine, an anaesthetic, has

been used in multiple randomized controlled clinical trials and exhibits strong antidepressant effects. Some studies have addressed its potential antianxiety properties but, so far, no known ongoing trials of ketamine in PD, GAD, or SAD have been reported.

Neuropeptides are small proteins that play an important role as neuronal signalling molecules, which are involved in an array of brain functions. Specific neuropeptides, such as oxytocin, substance P, neuropeptide Y, arginine vasopressin and cholecystokinin can modulate fear and anxiety. However, these findings have not yet been translated into novel pharmacological treatments and further research is still required.

Finally, cannabinoids are consumed for their euphoric and relaxing effects making them ideal for lowering anxiety and acting as relaxation inducers. They act on the cannabinoid type 1 (CB1) receptor, the serotonergic type 1A (5-HT_{1A}) receptor and the transient receptor potential vanilloid type 1 (TRPV1). While the activation of the CB1 receptor produces inhibitory effects leading to anxiolytic effects, high doses of CB1 receptor agonists induce activation of the TRPV1 receptor producing anxiogenic effects. Since drugs that act as 5-HT_{1A} receptor agonists have proved to be effective in the treatment of anxiety disorders, cannabinoids are strong candidates as anxiolytic agents, although the amount of evidence is still very low.

In summary, it is important to stress that, when taken together, the prevalence, comorbidity, and morbidity of anxiety and related disorders is exceptionally high. Despite its large cost burden compared to other psychiatric disorders, research on novel medication treatments over the past decade has been limited. The knowledge of genetic and epigenetic mechanisms of anxiety disorders can lead to a better comprehension of the neurobiology behind these diseases and to the development of more effective preventative and treatment strategies. Despite the potential impact of understanding these mechanisms, evidence based on human studies is still limited.

4.3. Schizophrenia

Schizophrenia is considered the most frequent psychotic disorder, affecting approximately 1% of the world population. It is associated with a heavy health care burden, with annual related costs in the United States estimated to be more than 150 billion dollars. People suffering from this disorder usually have a reduced life expectancy, about 15 years shorter than the general population, with a 5-10% lifetime risk of suicide. This disorder

affects men more frequently than women in a 4:1 ratio. It may be diagnosed at any age, but usually manifests itself in adolescence and early adult life. Several studies suggest that many patients have a history of lower intelligence quotients, with hearing, motor, emotional and social impairments in their childhood.

It has a heterogeneous spectrum of clinical manifestations with significant impact on social and occupational activities. The diagnosis of schizophrenia is based on clinical assessment with patients experiencing loss of contact with reality and impairments in thought, mood and behaviour. It is diagnosed in the presence of (1) psychotic symptoms, such as hallucinations, delusions or disorganized speech, and (2) negative symptoms, namely decreased motivation, reduced emotional expressiveness and cognitive impairments (executive functions, memory and speed of mental processing).

4.3.1. Pathophysiology and treatment – present and future

Schizophrenia has a complex multifactorial aetiology with environmental and genetic factors contributing to its onset. Family studies suggest an important impact of genetics in this disorder, with an estimated heritability of 80%. Genome-wide association studies identified multiple *loci* associated with schizophrenia, namely genes involved in regulation of the postsynaptic membrane, synaptic transmission, neurodevelopment and immune functions. However, even among identical twins, pairwise concordance for schizophrenia is only around 50%, highlighting the importance of environmental factors and their interactions with genes in the onset of schizophrenia.

Although some risk factors have been associated with schizophrenia, in isolation they do not appear to be significant. They include maternal infections, malnutrition during pregnancy, preterm birth and preeclampsia, suggesting that the pathogenesis of schizophrenia begins early in neurodevelopment. Increased paternal age, trauma, neglect, social deprivation and psychological distress are also associated with this disorder. Moreover, several studies point to the contribution of substance abuse to the onset of schizophrenia, particularly cannabis, which has a dose–response relationship between the extent of use and the risk of psychosis.

The pathophysiology of schizophrenia has been classically associated with the dopaminergic hypothesis. This theory suggests the role of an excessive dopamine activity in the onset of positive symptoms, acting through D_2-receptors localized in the limbic regions of the brain. Accordingly,

amphetamines and other drugs that release dopamine into synaptic clefts induce psychotic symptoms, in contrast to antipsychotic drugs that reduce positive symptoms by blocking D_2 receptors. Moreover, *in vivo* molecular imaging studies provide evidence that dopamine synthesis and release capacity is higher in patients compared with control participants and that amphetamine administration leads to a higher release of dopamine, directly associated with the worsening of psychotic symptoms.

Several studies have pointed out that subcortical dopamine activity dysregulation may be triggered by stressful life events, through aberrant reactions of neuronal circuits of stress response, namely the HPA axis, the amygdala and the PFC. These observations are consistent with a role of cortisol in the onset of psychosis. Mizrahi *et al*. (2012), investigating dopamine release in response to a psychosocial stress challenge in psychosis-related disorders, found higher stress-induced changes in salivary cortisol in the schizophrenia group than in the control group. Additionally, greater changes in cortisol response were associated with higher dopamine release in the associative striatum.

In addition to the classical dopamine theory of psychosis, the glutamate theory (specifically the NMDA hypoactivity theory) proposes that psychosis may also result from a reduced activity of NMDA receptors in the PFC. This theory is supported by the fact that dissociative anaesthetics such as phencyclidine and ketamine, which act as NMDA antagonists, lead to psychotic effects. It is thought that reduced activity of NMDA receptors on GABA interneurons in the PFC leads to the hyperfunctioning of downstream glutamate signalling to the ventral tegmental area, increasing dopamine release.

A third hypothesis, the serotonin theory, suggests that cortical serotonin hyperfunction can also contribute to psychosis. Lysergic acid diethylamide (LSD) and psilocybin, which act mostly as 5-HT_{2A} agonists, result in visual hallucinations and mystical delusions. This theory proposes that the hyperactivation of 5-HT_{2A} receptors on glutamate neurons promotes downstream release of glutamate in the ventral tegmental area, thus activating the mesolimbic pathway, by increasing dopamine levels in the ventral striatum.

Despite the three neurochemical hypotheses, all current licensed treatments for schizophrenia are D_2-receptor blockers. First-generation antipsychotics (FGA) were first developed for the treatment of schizophrenia in the 1950s, but they have also been shown to be effective in the treatment of other

psychiatric conditions, such as bipolar disorder and agitation. FGA are phenothiazine derivatives, which act by antagonizing post-synaptic D_2 receptors. Imaging studies show a high correlation between treatment effectiveness and a requirement of at least 65% D_2 receptor occupancy in the brain. Binding of FGA to dopamine receptors in several regions of the brain, namely the cortex and the striatal area, accounts for a risk of movement disorders, such as extrapyramidal symptoms, including tremor and akathisia, some of which appear after long-term exposure (tardive dyskinesia). Moreover, prolactin secretion in both sexes is largely controlled through the inhibitory effect of dopamine in the pituitary. Thus, FGA blockage of D_2 receptors present in the tuberoinfundibular circuit disinhibits the secretion of prolactin resulting in hyperprolactinaemia, which leads to several clinical manifestations, including gynaecomastia, galactorrhoea, sexual dysfunction, and infertility. Each FGA also exhibit varying degrees of dopamine (D_1–D_5), histamine, and cholinergic receptor blockage, which are associated with various side effects as shown in Table 5-3.

Second-generation antipsychotics (SGA) have comparable clinical efficacy in relation to FGA. SGA act by blocking D_2 receptors, with some exceptions, namely aripiprazole, which acts as a D_2 receptor partial agonist. However, most SGA have a higher affinity for serotonin $5HT_2$ receptors than for D_2 receptors, which is probably the reason for the lower overall risk of extrapyramidal side effects. Although these theories have not been fully confirmed, other aspects of SGA may contribute to this lower risk, namely higher dissociation rates of these drugs from D_2 receptors and a preferential binding to D_2 receptors localized in limbic and cortical regions, rather than in the nigrostriatal circuits. For these reasons, SGA are normally preferred for the treatment of psychotic disorders, in detriment of FGA. However, SGA also act as blockers or partial agonists of muscarinic, histaminic and α-adrenergic receptors, with secondary anticholinergic, sedative, metabolic and hypotensive side effects (Table 4-3). Each SGA has different affinity patterns for these receptors having unique side effects profiles.

Table 4-3. Mechanisms of action and side-effect profiles of antipsychotic drugs.

Antipsychotic generation drug	Types of drugs	Cell targets and molecular actions	Adverse effects
FGA	Haloperidol Chlorpromazine Levomepromazine	D_2 receptor antagonists Muscarinic receptor antagonists H_1 receptor antagonists α-adrenergic receptor antagonists	*Higher risk of:* Extrapyramidal symptoms (tremor, akathisia, slurred speech) Parkinsonism (bradykinesia) Tardive dyskinesia (movement disorder associated with age and time of exposure to the drug; involuntary choreoathetoid movements of the face, tongue, trunk and extremities) *Variable risk of:* Hyperprolactinaemia (gynaecomastia, galactorrhoea, sexual dysfunction, and infertility) Anticholinergic symptoms (dry mouth, constipation, urinary retention; higher risk with chlorpromazine) Sedation (through antagonism of H1 receptors; higher risk with chlorpromazine) Orthostatic hypotension (through antagonism of α-adrenergic receptors; higher risk with chlorpromazine) Metabolic syndrome (weight gain, diabetes, dyslipidaemia; higher risk in chlorpromazine; unknown physiological mechanism) QTc interval prolongation Increased risk of mortality (mechanism not firmly established)

			Neuroleptic malignant syndrome (fever, muscle rigidity with rhabdomyolysis, conscience alterations and autonomic impairment; medical emergency with unknown physiological mechanism)
SGA	Olanzapine Risperidone Paliperidone Quetiapine Ziprasidone Aripiprazole	D_2 receptor antagonists 5-HT_{2A} receptor antagonists <u>*Aripiprazole:*</u> D_2 receptor partial agonist 5-HT_{2A} receptor antagonist $5HT_{1A}$ partial agonist <u>*In variable extent:*</u> Muscarinic, α-adrenergic and H_1 receptor antagonists or partial agonists	*Higher risk of:* Metabolic syndrome (weight gain, diabetes, dyslipidaemia; higher risk with olanzapine; unknown physiological mechanism) *Variable risk of:* Hyperprolactinaemia (gynaecomastia, galactorrhoea, sexual dysfunction, and infertility; higher risk with risperidone and paliperidone) Anticholinergic symptoms (dry mouth, constipation, urinary retention) Sedation (through antagonism of H_1 receptors; higher risk with quetiapine) Orthostatic hypotension (through antagonism of alpha-adrenergic receptors; higher risk with quetiapine and paliperidone) Neuroleptic malignant syndrome Extrapyramidal symptoms (tremor, akathisia, slurred speech) Parkinsonism (bradykinesia) Tardive dyskinesia (movement disorder associated with age and time of exposure to the drug; involuntary choreoathetoid movements of the face, tongue, trunk and extremities)

Key: 5-HT, Serotonin; D, dopamine; FGA, First generation antipsychotics; H, Histamine; SGA, Second generation antipsychotics

D_2 receptor blockers fail to improve positive symptoms in about one-third of the patients. Thus, it is possible that dopamine antagonism is not directly related to the pathophysiology of treatment-resistant schizophrenia (TRS). Several clinical aspects have been associated with TRS including severe premorbid social functioning, young age at onset, longer standing untreated disease and a history of drug abuse. For these individuals, the drug clozapine appears to be of particular benefit, but the mechanism underlying this remains poorly understood. So far, no imaging, genetic or clinical markers have proved to be suitable for the diagnosis or prognostic estimation of schizophrenia. Lower striatal dopamine synthesis, higher glutamate concentrations and more pronounced grey matter reduction have been associated with TRS. Surprisingly, glutamatergic drugs have not shown promising results in treating these refractory disorders.

These observations suggest that schizophrenia is a heterogeneous psychiatric disorder with a wide spectrum of clinical and biological manifestations. Future research into the biology will hopefully identify biomarkers of this disease with significant implications for the diagnosis and personalized treatment of schizophrenia. Several clinical and molecular investigations have suggested that neuroinflammatory processes and immune dysregulation may play a role in schizophrenia pathogenesis. This disorder has been associated with changes of both pro- and anti-inflammatory molecules in the central nervous system and in the peripheral blood. It is interesting to observe that the most effective antipsychotic drug to date, clozapine, has anti-inflammatory properties, attenuates microglial activation and leads to long-term immune suppression, sometimes with undesired side-effects such as agranulocytosis and neutropenia. Brain imaging techniques have shown an increase in microglial activation in patients with schizophrenia, which is known to mediate the release of cytokines, namely IL1β, IL6, interferon-gamma (IFN_Y), S100B, TNFα. Accordingly, certain immunomodulatory adjunctive therapies have been shown to reduce psychotic symptoms in schizophrenia patients. Additionally, supplementary aspirin therapy has been found to reduce “Positive and Negative Syndrome Scale” scores, especially in early stages of the disease.

These and other studies will hopefully allow patient stratification based on molecular profiles, identifying individuals who are most likely to respond to particular drug interventions, thus improving clinical practice using personalized treatment strategies.

Conclusion

Psychiatric diseases are a major public health problem in developed countries. Due to the global lack of biomarkers in psychiatric disorders and the absence of objective tests, accurate diagnosis and selection of effective treatments remain challenging. Hence, psychiatric disorders are usually diagnosed based on clinical criteria and pharmacologic treatments are primarily chosen based on a 'trial and error' system. The use of precision medicine in psychiatry has been suggested as a promising strategy that could assist with improving patient treatment outcomes. Further investigation is required which takes genetic heterogeneity and the contribution of environmental factors into consideration, to develop treatments that are more individualised, effective and better tolerated.

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Chapter 5

Biochemical Changes in Nutritional Diseases

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List of abbreviations

ACTH: Adrenocorticotrophic hormone or adrenocorticotrophin or corticotrophin
ADP: Adenosine diphosphate
AGP: Alpha-1-acid glycoprotein
ATP: Adenosine triphosphate
BADGE.2H(2)0: Bisphenol A bis(2,3-dihydroxypropyl) ether
BMI: Body mass index
COPD: Chronic obstructive pulmonary disease
CRH: Corticotrophin releasing hormone
CRP: C-reactive protein
IBW: Ideal body weight
ICD-10: Classification of mental and behavioural disorders
DHEA: Dehydroepiandrosterone
DSM-5: Diagnostic and statistical manual of mental disorders
FSH: Follicle stimulating hormone
GABA: γ-Aminobutyric acid
GH: Growth hormone
ICNND: Interdepartmental Committee on Nutrition for National Defense
IGF-1: Insulin-like growth factor 1
IL: Interleukin

LH: Luteinizing hormone
NADH: Nicotinamide adenine dinucleotide
NGFIB: Nerve growth factor IB or Nur77
PAI-1: Plasminogen activator inhibitor-1
PTH: Parathyroid hormone or parathormone
T_3: Triiodothyronine
T_4: Tetraiodothyronine or thyroxine
TNF: Tumour necrosis factor
TSH: Thyroid stimulating hormone or thyrotrophin
VLDL: Very low-density lipoprotein

Introduction

Nutritional diseases are any of the nutrient-related disorders and conditions that cause illness. This group of diseases is an important public health problem, nowadays. These disorders occur when dietary intake does not contain the right amount of nutrients for healthy functioning of the body, or when nutrients from food cannot be properly absorbed.

Nutritional diseases include a wide range of conditions, such as widespread undernutrition (malnutrition), overnutrition that leads to obesity, and eating disorders. These diseases also include developmental abnormalities that can be prevented by diet, hereditary metabolic disorders that respond to dietary treatment, the interaction of foods and nutrients with drugs, food allergies and intolerances, and potential risks in the food supply.

It is known that inappropriate eating habits, such as under- or over-eating, or consuming many types of food and drinks with a low fiber content or a high fat, salt, and sugar content, can increase the risk of developing diseases and health problems, including cardiovascular disease, high blood pressure, sleep disorders, type 2 diabetes mellitus, osteoporosis, some types of cancer, and depression. Moreover, other diseases can be caused by a deficiency of specific micronutrients, such as vitamins and minerals.

Nutritional deficiencies can often worsen health outcomes (whether a disorder is present or not), and some disorders (e.g., malabsorption) can cause nutritional deficiencies. In addition, many patients (e.g., older patients during acute hospitalization) have unsuspected nutritional deficiencies that require treatment.

This chapter will focus on the biochemical changes that are present in nutritional diseases such as malnutrition in children and in the elderly,

obesity in children and adolescents and in the elderly, and eating disorders, principally in adolescents, such as anorexia nervosa, bulimia nervosa, binge eating disorder, and orthorexia nervosa and bigorexia.

5.1. Malnutrition

Malnutrition is a pathology that, nowadays is present especially in risk groups (children and the elderly). Malnutrition is the individual's nutritional status characterised by an insufficient intake of energy and nutrients, which results from the complex interaction between food, socioeconomic conditions, and health status. There are two main types of malnutrition: protein-energy malnutrition and dietary deficiencies.

Nutritional status influences the body's functionality, as well as the individual's well-being. This problem is a continuous process that focuses on inadequate food intake, either due to anorexia or food scarcity, decreased nutrient absorption capacity or increased losses and energy expenditure, followed by a decrease in anthropometric and biochemical values. Traceability and monitoring of nutritional status are vitally important to prevent malnutrition and apply a treatment early.

In the cycle of inadequate food consumption and development of disease (Fig. 5-1), several factors such as weight loss, mucosal damage, loss of appetite and malabsorption, aggravate the disease state, increasing the duration of recovery. This whole cycle contributes to the increase of malnutrition, as for example, a pathology in the mucosa of the gastrointestinal tract causes a malabsorption of important nutrients for the organism.

Metabolic changes can appear at any time in life, due to external causes. In malnutrition, depending on the degree, it is especially important to be aware of the impact of metabolic changes. Thus, it is necessary to use more precise nutritional methods to assess nutritional status, such as biochemical parameters. For example, the concentration of albumin and transferrin when combined become good indicators, but when isolated they can be late indicators of malnutrition. However, its interpretation is necessary considering the limitations, as its values can be masked due to an inflammatory state or in the presence of a pathology.

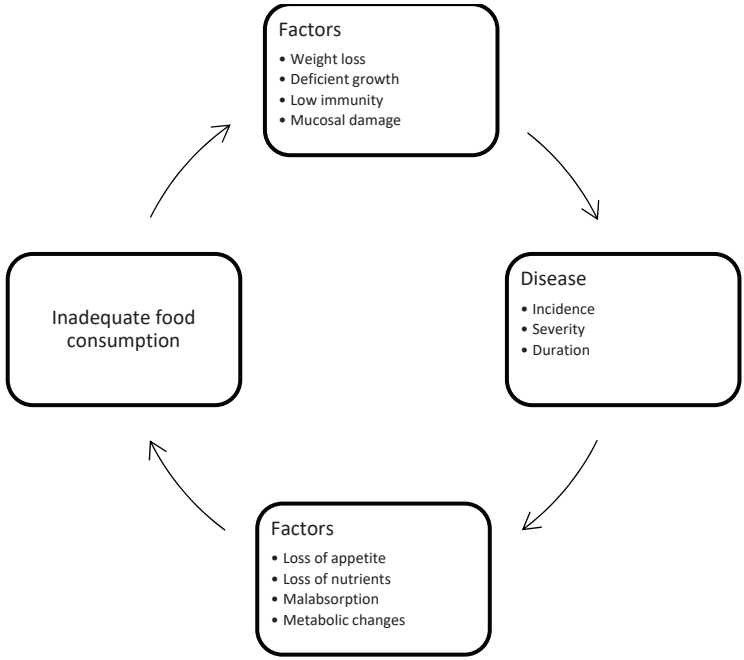

Fig. 5-1. Cycle of inadequate food consumption/increase in disease.

5.1.1. Malnutrition in children

Child malnutrition is a strong reality in developing countries. During growth, a varied, balanced, and complete diet is necessary, according to their nutritional needs. These parameters are not present in developing countries, due to external factors, such as scarce economic resources, social conflicts, eating disorders and pathologies that affect the correct and effective absorption of nutrients. When malnutrition occurs in the presence of a pathology, it is associated with a modified metabolism (decreased or increased), depending on the degree of the pathology.

The child has low levels of lean and fat mass, and therefore has lower reserves for resting energy needs. These factors alone or combined can trigger malnutrition, as the child has a higher nutritional need than the adult. Malnutrition interferes with the child's positive evolution, associated with the appearance of infections and the malfunction of vital organs, since the child is more susceptible, as his immune system is reduced.

Malnutrition is also present when the child stops breastfeeding and starts oral feeding. Breast milk is a natural food that provides all the energy and nutrients that the child needs, protecting against chronic infectious diseases and reducing malnutrition, growth problems and mortality.

Malnutrition in children can be classified as protein-energy deficiency. Protein-energy malnutrition is the set of pathological conditions resulting from simultaneous deficiencies, in varying proportions, of proteins and energy, which occurs more frequently in infants and young children and which is generally associated with infections. Protein-energy malnutrition has two severe forms: kwashiorkor and marasmus. These forms of malnutrition are considered serious due to the presence of important clinical and biochemical changes in nutritional status.

In Kwashiorkor there is a reduced intake of protein, which is lacking at various levels in the body, such as blood, peripheral tissues, muscles, and the liver, among others. Main symptoms of this condition are, among others, the appearance of edema, diarrhoea, depigmentation of the hair, apathy, sadness, and lack of appetite.

In the marasmus, energy intake is insufficient to meet energy needs. Therefore, the body uses energy reserves such as glycogen in skeletal muscle and, as a last resource, triglycerides which also occurs in protein-calorie malnutrition. The child with marasmus shows growth deficiency, as well as low weight, loss of fat and muscle mass and cachexia. The child also shows the appearance of an elderly person with wrinkled skin and has usually an irritated state.

Malnutrition in children can also be classified as acute or chronic. Chronic malnutrition is the relationship between height and age that leads to low growth. The plausible cause is maternal malnutrition before and during pregnancy or infant malnutrition during the first years of life, with a deficit in the macro and micronutrients essential for foetal/child development. Low growth increases mortality in the first years of life, and is associated with decreased cognitive, mental, and motor function. Consequently, the child will have low school performance and productivity. However, chronic malnutrition can be reversed until the age of two.

In turn, acute malnutrition is the relationship between height and weight, being defined as low weight for height. Acute malnutrition is likely to appear at any time in life and can be quickly recovered through correct food

choices and adequate health care. It is quite common to happen in the transition from breastfeeding to oral feeding.

5.1.2. Malnutrition in the elderly

Malnutrition, with the anorexia factor, is associated with an increase in quantitative malnutrition (for example, protein-energy malnutrition), due to inadequate nutrient intake. In the early stages, anorexia increases the risk of qualitative malnutrition, due to low intake of nutrients, such as proteins and vitamins.

Anorexia in the elderly is defined as loss of appetite and decreased food intake. It is present in about 15-20% of cancer patients at the time of diagnosis, being a side effect of that condition. Anorexia is clinically defined as loss of appetite or absence of the desire to eat. This condition is an important component of cachexia. On the other hand, cachexia is clinically defined as the loss of skeletal muscle and body mass (body mass index (BMI) below 16 kg/m^2). Cachexia is accompanied by persistent fatigue, due to the metabolic changes and advanced malnutrition.

Multifaceted clinical conditions are common in the elderly. Many of these clinical conditions are prevalent and related to numerous comorbidities and adverse effects, resulting in disability and low quality of life, such as sarcopenia. Sarcopenia is a syndrome characterised by progressive and widespread loss of muscle mass and skeletal strength, with the risk of increased falls, fractures, and death.

There are several mechanisms related to anorexia in aging, such as smell and taste that play an especially important role in eating and drinking. These two senses diminish over time during the life span, contributing to a decrease in food intake. Illness, medication, and smoking are some of the factors that can lead to changes in these senses. Therefore, it is necessary to encourage the elderly to increase their food intake by improving texture and taste, as well as increase the diversity in the diet and give assistance during meals.

Poor dentition and poorly placed dentures are also limiting factors in the consumption and amount of ingested food. Bad dentition influences the degradation of macro- and micronutrients, since the deficient formation of the bolus interferes with digestion and, consequently, leads to the malabsorption of nutrients, resulting in nutritional deficits. This problem (poor chewing) is associated with a lower intake of specific nutrients,

including fiber, vitamins, calcium, and proteins and, consequently, with a diet that is richer in fat.

There are other risk factors associated with anorexia in the elderly, such as psychological factors, medication, social and economic factors, lack of culinary practice/knowledge and several pathologies. One of the factors is the lack of vision and hearing that directly interfere with the ability to purchase, prepare, and consume food, being related to the reduction of food intake and loss of appetite. Depression is also very present in the elderly being associated with loss of appetite. These patients develop several symptoms, such as weakness, gastralgia and diarrhoea, that lead to loss of appetite and consequent anorexia.

Pathologies associated with the elderly, such as gastrointestinal diseases, malabsorption syndrome and chronic or acute infections, often cause micro- and macronutrient deficits and anorexia, a consequence of low intake. Conditions like cognitive heart failure, chronic obstructive pulmonary disease (COPD) and Parkinson's disease are often associated with anorexia and increased energy expenditure. As a result, changes in appetite frequently occur, which in turn lead to malabsorption or increased metabolic rate. In addition, if the patient is bedridden, morbidity occurs, which leads to the loss of lean mass and consequent weakness state.

Malnutrition is also a common problem among cancer patients, especially cancer of the pancreas, oesophagus, gastrointestinal and head and neck. Weight loss in cancer patients is often associated with loss of muscle mass and adipose tissue, which is different from loss induced by hunger. If left untreated, this muscle mass degradation can progress to severe loss associated with the anorexia-cachexia syndrome. This syndrome can be defined as a multifactorial disease with high lethality, capable of promoting several physiological changes, to adapt the organism to the scarcity of nutrients.

During hospitalization, involuntary loss of weight and muscle mass often occurs, being a determining factor for the development of hospital malnutrition, that increases morbidity and mortality. The period of hospital stay will be longer, increasing the susceptibility to infection scenarios, inherent in environments with multi-resistant bacteria.

Prescription of a drug-therapeutic strategy can also have many adverse effects, such as malabsorption, disorders of the gastrointestinal tract and loss of appetite. Anorexia can also result from drug-nutrient interactions and

their adverse effects. Cardiovascular drugs, psychic drugs, and other medications, such as the use of laxatives, can contribute to weight loss, causing malabsorption. To reverse this scenario, the evaluation of the pharmacological therapy used needs to be done and, if required, change the strategy.

Elderly with anorexia may show lack of the essential amino acid leucine and vitamin D, increasing, for instance, the risk of the occurrence of sarcopenia and fragility. Since vitamin D plays an important role in calcium fixation in bones, its supplementation (800 IU/day) increases the number of type 2 transversal muscle fibers (lost in individuals with sarcopenia), resulting in an increase in muscle mass and strength, thus reducing the risk of falls and injuries. When the intake of essential amino acids is insufficient, the intervention of supplementation may be necessary to neutralize any deficit and improve muscle mass. The recommended protein intake for an adult is 0.8 g/kg/day. However, in the elderly it should be increased to 4.0 g/kg/day due to a higher threshold to activate protein synthesis in muscles. It is important to note that proteins should be consumed throughout the day distributed over the various meals, to try to optimise the muscular anabolic response.

The regulation of appetite, when in deficit, is the key to understand elderly anorexia. Leptin and ghrelin are hormones that regulate food intake (Fig. 5-2). Ghrelin is the only hormone responsible for hunger stimulation, being gradually released by the cells of the stomach lining. There is no sustainable evidence on how ghrelin acts during aging, however, it is likely the existence of a relationship between leptin and the increase in circulating insulin, which may be related to a lower sensitivity to ghrelin, which results in a false satiety. On the other hand, leptin is responsible for controlling food intake, as it conveys the message of satiety, being released by the cells of the adipose tissue. It is involved in post-pandrial anorexia, as it is accompanied by increased plasma concentrations of post-pandrial insulin. Insulin regulates blood glucose levels and acts as a satiety hormone. The decrease in glucose tolerance and the levels of insulin in the circulation observed during aging can accelerate the development of anorexia. The action of insulin is indirect, increasing the anorexigenic signal from leptin to the hypothalamus and making it difficult to stimulate ghrelin.

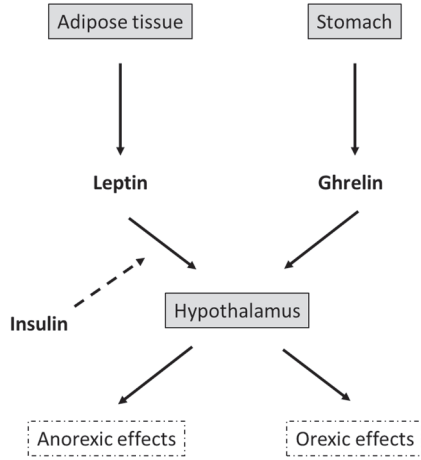

Fig. 5-2. Regulation of food intake by leptin and ghrelin.

5.1.3. Metabolic changes

Malnutrition causes several changes in biological systems, such as growth and development speed slowdown, changes in the nervous, endocrine, and immune systems and in the function of the pituitary gland, as well as metabolic adaptations like reduced basal metabolism and body temperature, and adjustments of skeletal muscle fibers.

Metabolic changes can appear at any time in life, due to external causes, resulting in increased energy and protein needs and in malabsorption of nutrients. These metabolic changes are varied and can occur at the level of macro- and micronutrients. One of these changes is the decrease in basal metabolism, which results from the decrease in lean mass. When the decrease in energy expenditure is not enough to compensate for the decrease in energy intake, body fat starts to be used, resulting in a decrease in adiposity and body weight. Lean body mass is also used, but decreases at a slower rate, due to the catabolism of muscle proteins used as energy supply. When the energy deficit becomes more severe, subcutaneous fat is used and is consequently drastically reduced. Protein catabolism leads to muscle atrophy. Various metabolic complications can occur in malnutrition, with emphasis on hypoglycaemia, hyperglycaemia, hypothermia, and dehydration.

In carbohydrate metabolism, glucose and lactose intolerance occurs (due to a decrease in lactase activity). Variations in glycaemic levels occur due to

excessive glucose production by gluconeogenesis mainly in liver cells, that results from reduced insulin synthesis, stimulated glucagon production and increased circulating adrenaline. Since the glycogen stores are rapidly consumed, glucose starts to be produced from free amino acids and glycerol, through the increase in gluconeogenesis. With the intensification of this alternative glucose producing pathway and the continuous decrease of macronutrient stores, it is difficult to control serum glucose levels, resulting in hypoglycaemia. There is also a decrease in adenosine triphosphate (ATP) and creatine phosphate reserves and an increase in free adenosine diphosphate (ADP). Malnutrition also causes changes in skeletal muscle such as decreased enzymatic activity of the respiratory chain, as well as decreased production of nicotinamide adenine dinucleotide (NADH) by the Krebs cycle, reduction in oxidative phosphorylation, and consequent change in oxygen consumption by the mitochondria.

In lipid metabolism, the increase in cortisol secretion promotes the excessive release of free fatty acids coming from the degradation of triglycerides (lipolysis) and the decrease in lipoprotein lipase activity, that result in insulin resistance and hyperglycaemia. The decrease in glycogen and fat stores reduces energy reserves, giving higher importance to protein mass, as this becomes the main source of energy.

Concerning protein metabolism, its degradation increases, being correlated with the modification of muscle fibers. Malnutrition can cause a decrease in the number of type 2 muscle fibers, as well as a decrease in their diameter. Proteins are important macromolecules in the body due to their structural functions, their action as biological catalysts, as well as their role in the immune function, transport of substances and regulators of cell development. When proteins cease to perform their structural or enzymatic role, the metabolic imbalance sets in causing protein-energy malnutrition, since, on a large scale, they are linked to biological homeostatic relationships.

In malnutrition, deficiencies are present not only in terms of macronutrients but also in micronutrients. During the acute phase response to malnutrition, pro-oxidant cytokines are produced which increase the use of antioxidant vitamins, such as vitamin A (retinol), C (ascorbic acid) and E (tocopherol) to compensate for oxidative stress. The use of minerals, such as iron, selenium, zinc, copper, and magnesium, is also increased to produce antioxidant enzymes. Studies have shown that malnourished individuals have decreased levels of the B vitamins, particularly B_2 (riboflavin), B_6 (pyridoxine) and B_{12} (cobalamin).

When both deficiencies (in macro- and micronutrients) are evident, dysfunctional changes in the immune system occur, such as higher production of free radicals and reduced synthesis of enzymes/proteins, leading to the development of viral or bacterial infections that can aggravate the state of malnutrition. The immune reaction to malnutrition, in response to infection, causes increased metabolic rate that promotes an increase in protein catabolism as well as change in the level of fatty acids.

5.1.4. Symptoms and diagnosis

Signs and symptoms related to malnutrition may be low growth and changes in behaviour (irritation, anxiety, and lethargy). Topical signs (thin and fragile hair, pale/swollen face, half-moon face), in the eyes (red membranes) and lips (bedsores), on the tongue (oedematous), teeth (cavities), skin (xerosis), subcutaneous tissue (oedema and low percentage of fat mass), cardiovascular (tachycardia), gastrointestinal (hepatosplenomegaly) and in the nervous system (mental confusion) may also be present.

The diagnosis of malnutrition is usually made by weight loss, anthropometric assessment, biochemical laboratory analysis and clinical and dietary aspects. These changes can be present isolated or in association, being identified a malnutrition case when at least two compromised nutritional parameters are present.

The simplest and most used method for assessing malnutrition is the anthropometric assessment, applicable to all age groups, which makes it more advantageous and inexpensive. Anthropometric measures must be interpreted with a keen critical spirit, since it is liable to misleading in cases of oedema, ascites, among others. On the other hand, weight is a good indicator for assessing nutritional status in children and the elderly. However, its real value can be masked by the presence of edema or dehydration, and in severe conditions and bedridden situations, given the difficulty in measuring body weight.

Skin folds are a measure of adiposity that allows the assessment of body composition, being an easy and effective technique for determining body fat. The most common are the tricipital, bicipital, subscapular and suprailiac skin folds (out of the wide range of 93 anatomical sites available for skin fold measurement). Subcutaneous fat constitutes a large part of total body fat and its amount varies depending on sex, age, and degree of adiposity. The folds can be correlated differently with the total body fat and the percentage of fat depending on the measurement site. For example, the

tricipital skinfold has a good correlation with the percentage of body fat. In males, it is the most relevant skinfold, being a good indicator of energy reserves, well correlated for all ages. There are limitations when there is oedema, that is, the oedema will give rise to a thicker fold, resulting in a false value, which leads to an incorrect assessment of nutritional status.

The average arm circumference is a good indicator that can be used alone or associated with the tricipital skinfold to assess nutritional status.

5.1.5. Biochemical parameters

In assessing nutritional status, biochemical parameters are a complement to food history, physical and anthropometric tests. The most used biochemical parameters in clinical practice, and more relevant for the study of malnutrition in any age group, are albumin, pre-albumin, retinol-binding proteins, transferrin, creatinine, 3-methyl histidine, excretion of urea, nitrogen balance, serum cholesterol and minerals.

Biochemical parameters are increasingly present in the early detection of protein deficiencies, however not all these parameters have the necessary and credible sensitivity for the evaluation. For instance, albumin is a good indicator for the diagnosis of severe malnutrition, however, has low sensitivity for the diagnosis of malnutrition in the early stages. There are also several proteins in the human body related to the metabolic response, however they can suffer alterations caused by factors other than changes in nutritional status, such as C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP), among others.

Albumin is a transport protein that is present in plasma, being one of the most used in the assessment of nutritional status. The low serum albumin concentration can be related to the increased incidence of associated comorbidities. Albuminaemia, according to several authors, portrays visceral protein reserves, which are distinct from somatic protein stores represented by skeletal protein mass. However, the visceral protein reserves undergo a lot of changes compared to the somatic protein reserves that remain within the normal limit. This demonstrates the big sensitivity of albumin synthesis to the protein content that comes from the diet. The Interdepartmental Committee on Nutrition for National Defence (ICNND), established the following ranges of albuminemia: deficient < 2.8 mg/dL; normal = 2.8-3.5 mg/dL and acceptable > 3.5 mg/dL.

In kwashiorkor, hypoalbuminemia has been considered a biochemical abnormality in children with severe protein deficiencies, because its value is exceptionally low. In marasmus, this reduction is not so significant since the vital function of essential tissues is preserved. In children with marasmus, their own tissues are consumed so that the body can obtain the necessary nutrients for energy homeostasis. The main factor of the low sensitivity of albumin in the diagnosis of the acute phase of protein-calorie malnutrition is its biological half-life (+/- 20 days), because after several weeks there may be changes in the response to variations in protein intake. External factors can affect their values, such as situations that alter the liver compartment of protein or energy substrates.

Pre-albumin is a glycoprotein of hepatic synthesis with a serum concentration up to 100 times lower than albumin. Its function is to transport thyroid hormones. The levels of pre-albumin are reduced in energy-protein malnutrition but can be restored to normal during nutritional intervention. This is because pre-albumin is synthesised by the liver and catabolised in the kidneys. Pre-albumin decreases in cases of infection and liver failure, unrelated to nutritional status, in response to cytokines, increasing in cases of kidney failure. As its life span is short (about two days), it is a good indicator to assess nutritional changes, because it has great sensitivity to organic changes. However, when used, special attention is needed since its value may be low due to infections.

Transferrin is a plasma β-globulin protein that is present in the liver cycle. It is an essential protein for the transport of iron between cells. It is considered effective in assessing nutritional status, being more advantageous than albumin due to its shorter half-life (about 8 days), which makes it more sensitive to changes in protein synthesis. Transferrin values can be affected by several factors during liver synthesis, such as iron deficiency, infection, kidney disease and heart failure. Serum transferrin levels below 170 mg/dL correspond to moderate protein deficiency and lower than 150 mg/dL to severe deficiency.

Retinol-binding proteins, as they have a noticeably short half-life (12 hours), are also good indicators for the nutritional status due to their sensitivity to changes in nutritional status. However, these proteins have no relevance for nutritional assessment of renal patients since their levels increase with the intake of vitamin A and decrease in cases of liver disease.

One of the important biochemical parameters is the measurement of muscle proteins. It is known that the muscle consists of 80% water and 20% protein.

Knowledge about this tissue is important in assessing nutritional status. Creatinine is found mainly in the skeletal muscle and measures skeletal muscle catabolism. When this molecule is formed, it is excreted via the kidney at a constant rate, regardless of enzymes. Therefore, urinary creatinine excretion may be related to the individual's muscle mass. When the diet is free of creatinine, the maximum exponent of total creatinine and the average concentration per kg of muscle remains constant. In critically ill patients, the creatinine index is an effective evaluation parameter for detecting malnutrition. Conditions such as age, the quantity of ingested proteins and the renal function itself are factors that influence this index and the consequent poor assessment of malnutrition.

3-Methyl histidine is a non-reusable amino acid for protein synthesis. It is assumed as an evaluation parameter since it is a metabolite of the muscular component derived from the catabolism of myofibrillar proteins. In the elderly and the malnourished, the content of this molecule increases in hypermetabolism. The excretion of urea is also assumed to be a measure of protein catabolism, varying in accordance with the intravascular volume, renal function, and nitrogen supply. The nitrogen balance is a good parameter for assessing protein intake and degradation, being relevant in the assessment of patients with malnutrition. This balance is determined by a non-invasive and low-cost technique, being a widely used parameter. The nitrogen balance corresponds to the difference between the introduced and excreted nitrogen, to assess metabolic stress.

When serum cholesterol is lower than 160 mg/dL, it may indicate a state of malnutrition, however a reliable value is only obtained after the disease is already well stablished, being, for this reason, a limitation in the assessment of the nutritional status. Low serum cholesterol levels are also observed in liver failure and malabsorption.

Minerals such as Fe, Zn, Cu, Mn, Cr, Se, among others, are essential to maintain good nutritional status, being found in amounts less than 100 mg/kg of total body weight. The serum levels of some minerals can be used to assess nutritional status, but external causes such as oedema, kidney function and trauma can lead to low plasma values, making the results less plausible. As an example, an individual in a hypercatabolic state shows an increase in urinary zinc excretion.

5.2. Obesity

The terms obesity and overweight refer to excess body weight in relation to height. Its definitions are arbitrary and are based on estimates of ideal body weight (IBW), that is, body weight associated with lower morbidity and mortality. The relative weight is the body weight in relation to the IBW. Overweight is defined as a relative weight of up to 20% above the IBW and obesity is a relative weight of 20% above the IBW. BMI is well correlated with body fat measurements and is defined as weight (kg) divided by height^2 (m^2). Overweight is defined as a BMI of 25-30 kg/m^2 and obesity as a BMI > 30 kg/m^2. The thickness of skin folds is also a measure of body fat stores.

The cause of most obesity cases is not known. Endocrine disorders, such as hypothyroidism or Cushing's disease, are rare causes. In obesity, genetic factors interact with environmental facts. As a genetic contribution, it is known that 80% of children with two obese parents will be obese, while only 14% of the children of two parents with normal weight will be obese. The main mechanism of weight gain is the consumption of more calories than daily energy needs. The treatment of obesity involves dietary restrictions, increase in physical activity and behaviour changes.

5.2.1. Obesity in children and adolescents

Obesity in children and adolescents is a growing concern in developed and developing countries and affects all socioeconomic groups. This is one of the critical life periods for the onset of obesity. Approximately 70% of obese adults started gaining weight in their teens. It has become a serious public health problem that affects quite a significant number of individuals in the world population. The proportion of overweight children and adolescents is increasing, being estimated that more than 22 million children under the age of 5 are obese. An increase in the consumption of total fat, saturated fat, cholesterol, sugar, and salt is also observed worldwide, leading to an increase in the prevalence of obesity in adolescents. The decrease in physical activity also contributes to obesity, especially in adolescents living in urban areas.

Appetite increases during adolescence and sedentary individuals are more likely to accumulate fat if they have access to high-calorie foods. Thus, a low level of activity among adolescents is a key factor for the worldwide increase in obesity in this period. Overweight in childhood and adolescence is associated with several immediate and long-term risks, including increased cholesterol, triglycerides and glucose, high blood pressure, type 2

diabetes mellitus, and a high risk of developing obesity in adults and its associated consequences.

Obesity is considered a multifactorial characteristic. Parents' food choices directly influence children's food preferences. In addition, it has been proven that suboptimal cognitive stimulation at home and low socioeconomic status contribute to the development of obesity, since it leads to the consumption of less fruits and vegetables and a higher intake of saturated fats. The risk of becoming obese also increases with the short duration of sleep, which leads to an increase in body fat.

In addition to the described environmental factors that influence the development of obesity, several genes may be involved and among them are the genes that encode leptin or its receptor, β-adrenergic receptors, and glucocorticoids, as well as the Na^+-K^+-ATPase pump. For instance, mutations in the gene encoding leptin, that lead to its deficiency, have been found in obese individuals. In addition, a mutant leptin receptor has also been identified in severe early-onset human obesity.

Body composition during puberty is a marker of the metabolic changes that occur during this period of growth and maturation. During puberty, there is an increase in body composition (total body fat, lean body mass and bone mineral content). In addition, adipose tissue is endocrinologically active and is centrally involved in the interaction between adipocytokines, insulin and sex steroid hormones and therefore influences the cardiovascular system and metabolic disease processes. The composition of the pubertal body is important, not only for the assessment of the current nutritional status, but also because it is directly associated with the possible appearance of a chronic disease later in life.

Body weight and energy expenditure are controlled by several complex endocrine interactions that directly influence food intake. The main molecule involved in this regulation is leptin, a hormone produced by adipose tissue that acts in the hypothalamus (Fig. 5-2). Leptin is a sensor for the presence of sufficient fat stores in the body and therefore suppresses food intake. During fasting or weight loss, plasma leptin and insulin levels are low, leading to the stimulation of neuropeptide Y synthesis and a consequent increase in food intake and decreased energy expenditure. Under these conditions, the synthesis of ghrelin, produced in the stomach, will be stimulated, leading to increased appetite. On the other hand, during food or weight gain, leptin and insulin levels are high, triggering a decrease

in food intake and an increase in energy expenditure. This is controlled by the launch of the corticotrophin releasing hormone (CRH).

Obesity is clearly associated with several health complications that culminate in an increased risk of cardiovascular disease. Excessive fat deposition in adipose tissue, especially in the abdominal region, is one of the contributing factors. Abdominal adipocytes are more resistant to insulin and, being closer to the portal circulation, release large amounts of free fatty acids that can lead to increased very low-density lipoprotein (VLDL) production and gluconeogenesis and decreased degradation of insulin. Together, these changes contribute to insulin resistance and hyperinsulinemia, which lead to hypertension and trigger the atherosclerotic process. In addition, obese adolescents tend to have high blood levels of glucose, insulin, total cholesterol, leptin, homocysteine, and CRP.

5.2.2. Obesity in the elderly

Ageing is associated with an almost linear decline in the basal metabolic rate. Skeletal muscle is a fundamental organ that consumes most of the energy produced in the healthy human body. The total volume of skeletal muscle can be estimated by 24-hour creatinine excretion. With aging, the volume of skeletal muscle decreases and the percentage of adipose tissue increases. It has been shown that the reduction in muscle mass in relation to the total body can be totally responsible for the decrease in the basal metabolic rate associated with age.

Changes in skeletal muscle also lead to reduction in energy consumption by physical activity. Consequently, the elderly have decreased energy needs. With the reduction of energy expenditure, the intake of essential nutrients also decreases. On the other hand, if energy intake exceeds individual needs, fat accumulates in the body. In the elderly, body fat tends to accumulate in the abdomen and the adipose tissue in the abdominal cavity is directly connected to the liver through the portal vein.

During ageing, subcutaneous fat decreases, while visceral fat accumulates. This accumulation of abdominal fat causes disturbances in the metabolism of glucose and lipids. Visceral and subcutaneous fat are biologically distinct in terms of gene expression and secretory profiles of adipokines and proinflammatory cytokines, such as leptin, tumour necrosis factor α (TNFα), interleukin-6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) (Fig. 5-3). In addition, the expression of adipokines in adipose tissue is regulated by nutrients and this response is exacerbated with ageing. Another

potential complication of visceral fat accumulation is the increased release of free fatty acids that can reach the liver through the portal circulation and interfere with the action of insulin in the liver.

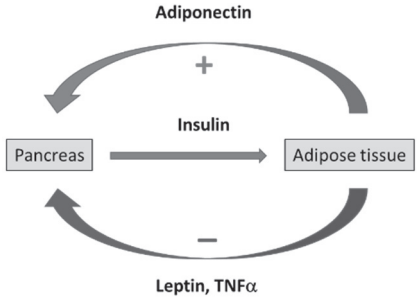

Fig. 5-3. The adipo-insular axis.

During ageing, lean body mass decreases and body fat increases. The percentage of body fat correlates positively with fasting blood glucose, insulin, and glucagon. Older individuals can have an impaired glucose counter-regulation to hypoglycaemia, associated with higher plasma insulin and reduced glucagon levels. Decreased physical activity and a high-carbohydrate diet impair glucose tolerance. In addition, there may be gender-related changes in glucose metabolism with age. Healthy elderly men have an impairment in non-oxidative glucose metabolism, but women do not. Glucose tolerance has been shown to decrease with age. More than 50% of adults over 80 years of age have glucose intolerance. The progressive reduction of insulin secretion by pancreatic β-cells, the increase in peripheral glucose resistance due to physical inactivity, the accumulation of abdominal fat and the decrease in lean mass contribute to the deterioration of glucose metabolism.

The whole body's sensitivity to insulin and the intracellular glucose oxidation rate is reduced in the elderly when compared to young adults. Possible explanations for reducing insulin effectiveness with ageing include increased abdominal fat mass, decreased physical activity, sarcopenia, mitochondrial dysfunction, hormonal changes (lower insulin-like growth factor 1 (IGF-1) and dehydroepiandrosterone (DHEA)) and increased oxidative stress and inflammation. However, insulin sensitivity decreases with age, even after adjusting for differences in adiposity, fat distribution and activity.

Genetic factors and obesity contribute to the development of insulin resistance, which is behind type 2 diabetes mellitus. Obesity contributes to this condition, since large visceral fat adipocytes are less sensitive to insulin and because the adipose tissue secretes hormones, adipokines, which regulate the insulin sensitivity of skeletal muscles. Thin elderly patients with type 2 diabetes mellitus have a profound impairment in insulin release and a slight resistance to insulin-mediated glucose clearance. In contrast, obese elderly patients with this disorder exhibit significant resistance to insulin-mediated glucose clearance, but adequate circulating insulin. In both groups, hepatic glucose production does not appear to be increased. On the other hand, insulin resistance increases due to the accumulation of common visceral fat in the elderly, reduced adiponectin, increased CRP, IL-6, TNFα, leptin resistance and intracellular triglycerides and mitochondrial dysfunction (Fig. 5-3).

Insulin resistance represents an important feature of the metabolic syndrome and is commonly observed in the elderly. The main deficiencies observed include unrestricted hepatic gluconeogenesis, adipose lipogenesis and deficient glycogen synthesis and glucose uptake by skeletal muscle. Abdominal obesity is a major contributor to insulin resistance and metabolic syndrome.

Metabolic syndrome, also called syndrome X or insulin resistance syndrome, refers to metabolic abnormalities such as hyperglycaemia, hypertension and dyslipidaemia that increase the likelihood of developing cardiovascular disease and type 2 diabetes mellitus, due to metabolic relaxation. Risk factors for metabolic syndrome include advanced age (> 70 years), obesity (particularly central obesity), family history of cardiovascular disease, type 2 diabetes mellitus, post-menopausal status in women, hypogonadism in men, smoking, consumption of a high carbohydrate diet, physical inactivity, and obstructive sleep apnea. Visceral obesity is considered crucial for the pathophysiology of the metabolic syndrome. The lipocytes found in the central fat deposits produce pro-inflammatory mediators, such as resistin and adiponectin, which increase insulin resistance in the elderly.

The prevention or treatment of obesity is the basis for the management of the metabolic syndrome. Treatment strategies mainly involve educating the patient about the importance of lifestyle changes to reduce weight, increase regular physical activity, reduce dyslipidaemia, lower blood pressure, and normalize plasma glucose levels. Adherence to healthy eating patterns has a significant impact on cardiometabolic and endocrine biomarkers, as these changes in lifestyle improve the patient's BMI. Pharmacological treatment

for diseases such as hypertension, diabetes mellitus and dyslipidaemia is also used. Current guidelines emphasize the importance of adopting strategies related to diet and exercise for the treatment of glucose intolerance.

5.3. Eating disorders

Eating disorders are generally seen as complex psychiatric illnesses. This idea probably stems from the large amount of physical and psychological comorbidities associated with these cases. Eating disorders can develop along with other psychiatric conditions, such as depression, drug abuse or anxiety. These disorders usually develop during adolescence or early adulthood, however, there are cases of onset in childhood or even later in adulthood.

The rapid growth experienced in adolescence is associated with a greater demand for energy, proteins, vitamins, and minerals. The growth spurt requires rapid tissue expansion with special nutrient requirements, including amino acids for the growth of striated muscle, as well as calcium and vitamin D for bone development. The needs for energy and nutrients must correspond to the needs of adolescents, since they usually perform physical work or recreational exercises (on average, boys more than girls), which benefits the growth of striated muscle mass. The caloric requirement of male adolescents is greater than that of females due to the greater increase in height, weight, and lean mass.

Inadequate eating habits are common among teenagers. In fact, the prevalence of unsatisfactory nutrition is higher in this group than in any other age group. Failure to achieve optimal nutrition can lead to delayed linear growth and impaired organ remodelling. Iron deficiency is the main cause of anaemia in children and adolescents. Eating habits are affected by several factors during adolescence. Examples are environmental factors, such as the parental model, the influence of other teenagers and cultural beliefs, personal factors such as food preferences, body image and personal beliefs, and factors of the macrosystem, including types of food available, food production, mass media and announcement. In addition, teenagers are more likely to eat snacks and fast food or to diet than children. Teenagers also tend to acquire other unhealthy behaviours, such as smoking and consuming alcohol and drugs. All the mentioned factors can in some way influence the eating habits of individuals and can lead to eating disorders.

5.3.1. Anorexia nervosa

Anorexia nervosa is an eating disorder characterised by extremely low body weight, along with a strong fear of gaining weight and distorted weight perception. The risk of developing anorexia is higher in adolescents due to the changes that their body undergoes during puberty. It is estimated that about 0.3% of adolescents aged 13 to 18 years old suffer from anorexia, being more common in girls. However, anorexia can develop in individuals of any age, although it is rare in people over 40.

Anorexic individuals usually restrict the amount of food intake. Affected individuals also control body weight through excessive physical exercise but continue to consider that they are overweight and fear weight gain. In the eyes of the anorexic individual, thinness is equated with self-worth.

The cause of anorexia is unknown, but it is believed to be a combination of several factors, including biological, psychological, and environmental. As biological factors, some genes can be associated with the development of anorexia and certain genetic changes can increase the susceptibility to the development of this condition in some individuals. Some individuals also have a genetic tendency towards perfectionism or perseverance, which are characteristics associated with anorexia. Individuals with high levels of anxiety or who suffer from depression can also use dietary restrictions to try to reduce it. An environmental factor is the culture of thinness, often associated with success and value. Social pressure can trigger the adoption of anorexia, especially among female adolescents. Studies show that many teenagers, especially women, take inadequate doses of vitamins and minerals.

It can be difficult to diagnose this condition, as low body weight is different for each person and affected individuals often disguise their thinness, eating habits and physical problems. The signs and symptoms of anorexia can include intensive weight loss, thin appearance, altered blood counts, fatigue, dizziness and fainting episodes, insomnia, fingers with bluish discoloration, very thin hair that breaks and falls easily, skin becoming yellow and dry, irregular heartbeat, absence of menstruation, abdominal pain associated with constipation, low blood pressure, intolerance to cold, dehydration and swollen arms and legs.

Anorexia triggers several medical complications resulting directly from weight loss and malnutrition. One of the first consequences of massive weight loss is at the level of the skin, which becomes very dry and can easily

crack and bleed, especially in the fingers and toes. Due to prolonged starvation, the catabolism of macromolecules such as fat and proteins is induced, leading to loss of volume and cellular function. This leads to adverse consequences on the heart, brain, liver, intestines, kidneys, and muscles, including their atrophy. Once weight loss reaches 15-20% of ideal body weight, gastroparesis often develops, that is, a delay in emptying the stomach, along with swelling, pain in the upper quadrant and a feeling of early satiety. Constipation is also a frequent complication. In addition, most anorexic patients (95%) have bradycardia and hypotension and may develop pulmonary emphysema and decreased lung function.

Anorexia also has adverse effects on the bone marrow, probably affecting red blood cells, white blood cells and platelets. Consequently, anaemia, leukopenia and thrombocytopenia are likely to occur in these patients. However, predisposition to frequent infectious diseases is not common. In addition, anorexia has been shown to be associated with cerebral atrophy. In patients with severe cases of anorexia, the brain is not distinguishable from the brain of an Alzheimer's patient. Anorexic individuals become unable to concentrate, write or have sustained reasoning. Recent studies show that these changes are not completely restored after weight gain.

These patients also experience several hormonal changes. They have low levels of gonadotrophins and deep oestrogens deficiency. Some individuals have the lowest levels of luteinizing hormone (LH) seen in secondary amenorrhoea. Androgens secretion is deficient, indicating that gonadal sources are compromised. Anorexic patients also have low levels of oestradiol because of lack of ovarian stimulation. Moreover, leptin, a hormone produced by adipose tissue as a sign of the brain's nutritional status (Fig. 5-2), is also low in anorexic patients. On the other hand, ghrelin, produced in the stomach to increase appetite (Fig. 5-2), is present in high levels in this condition. In addition, adiponectin is also increased and appears to be inversely related to BMI and directly associated with increased insulin sensitivity (Fig. 5-3), generally seen in anorexia. Insulin levels are usually normal.

Other hormones that undergo changes in this condition are triiodothyronine (T_3) and tetraiodothyronine or thyroxine (T_4), which are low, while reverse T_3 is increased. However, thyroid stimulating hormone or thyrotrophin (TSH) levels are usually normal or slightly reduced, which suggests that the source of suppressed thyroid function is the hypothalamus (Fig. 5-4). The adrenal gland produces high levels of cortisol and high levels of CRH are also seen. However, the response of the adrenocorticotrophic hormone or

adrenocorticotrophin or corticotrophin (ACTH) is low, suggesting a feedback effect at the pituitary level (Fig. 5-4). Growth hormone (GH) is also high, along with reduced IGF-1, which suggests the acquisition of GH resistance.

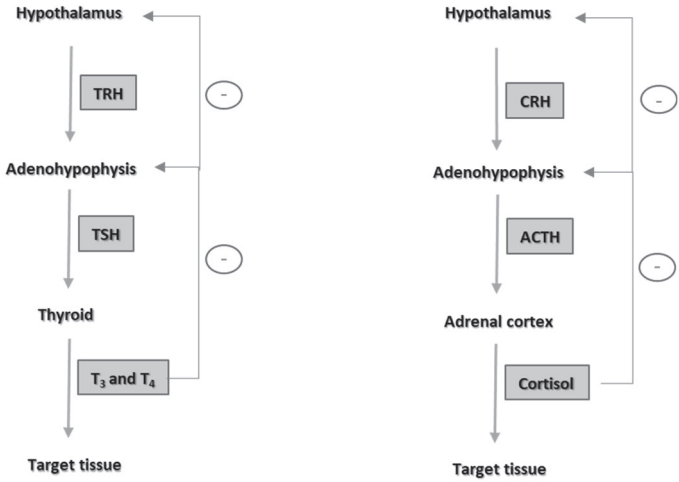

Fig. 5-4. Regulation of the secretion of T_3, T_4 and cortisol hormones.

Several of the endocrine changes described above, such as low levels of T_3, oestradiol, testosterone and IGF-1, and a high concentration of cortisol, are likely to be involved in the development of osteoporosis, a serious complication of anorexia. Excess cortisol inhibits bone formation, contributing to bone loss, while adiponectin seems to indirectly affect bone formation induction. In addition, androgens have an anabolic effect on bones, but because they are low in anorexia, they contribute to the loss of bone mass. Therefore, the loss of bone mass in anorexia has, in fact, a multifactorial origin. Levels of vitamin D and parathyroid hormone or parathormone (PTH) are generally normal in these patients.

5.3.2. Bulimia nervosa

Bulimia nervosa is an eating disorder characterised by consuming a large amount of food in one session, followed by an attempt to prevent weight gain by purging what was consumed. It has a destructive pattern of eating a

lot of food followed by self-induced vomiting, accompanied by serious emotional problems that, together, lead to life-threatening conditions. It is estimated that 4-13% of adolescents aged 13-18 years have bulimia. Patients with this condition tend to be older than those with anorexia and it is more common in men than anorexia.

Unlike anorexia, bulimia does not necessarily involve significant weight loss, but it also leads to serious health complications. This condition is usually associated with mental disorders and patients may experience depression, anxiety, obsessive-compulsive behaviours that can lead to suicide or alcohol/substance abuse. The lack of vitamins in these patients leads to bad mood and irritability. They are focused on weight control and can do compulsive physical activities.

Purging leads to generalised weakness and fatigue. Patients suffer from a sore throat and stomach and their teeth are damaged over time due to the acidic content of the vomit, with erosion of the enamel and tooth sensitivity. The acid can also irritate the esophagus and even cause it to rupture, causing bleeding. It can cause stomach pains, acid reflux and damage to the intestines, causing diarrhoea or constipation. Excessive use of diuretics and laxatives by these patients can cause kidney damage and lead to the development of haemorrhoids.

Dehydration is also a common symptom resulting from frequent purging. This can cause electrolyte imbalance, such as sodium, potassium, chloride, and bicarbonate, which results in change in plasma pH. Severe vomiting leads to hypokalaemia and other metabolic changes include hypocalcaemia, hypophosphatemia, and hypomagnesaemia, which can result in arrhythmias and, in extreme cases, can contribute to kidney problems and lead to heart failure. Bulimia patients may also have low blood pressure and anaemia.

Among the biochemical changes assessed in patients with bulimia, metabolic defects, such as abnormalities in the homeostasis of proteins, glucose, lipids, and enzymes are relatively common. Several studies have shown that increased serum levels of biochemical parameters are significantly related to bulimia. In addition, some studies showed that cytokines play a critical role in eating disorders and pro-inflammatory cytokine levels, such as IL-6, TNFα and IL-1β, have an increasing trend in patients with bulimia.

As in anorexia, these patients develop hormonal imbalance with change in the daytime leptin secretion, which tends to have a progressive and less than

normal drop during the day. This change is associated with exaggerated insulin secretion, high fasting blood glucose and, as expected, with increased levels of ghrelin. Thyroid dysfunction is also observed, with decreased levels of T_3 and T_4 that result in a reduction in the resting metabolic rate. Patients also have low levels of LH and follicle stimulating hormone (FSH), along with low levels of oestradiol. Experienced hormonal changes can interfere with regular menstruation or even stop it.

5.3.3. Binge eating disorder

Binge eating is characterised by recurrent episodes of eating large amounts of food over a short period of time, such as eating the amount normally eaten in an entire day in just two hours. These patients experience a sense of loss of control during episodes of binge eating and guilt afterwards. These individuals eat much faster than normal, only stop eating when they are uncomfortably full and eat even when they are not hungry. This is one of the newest officially recognized eating disorders.

Binge eating affects 2.2 to 4.6% of the general population and between 10 to 20% of obese people. It is associated with obesity and comorbidities, including psychiatric pathologies. It usually contributes to obesity, as patients have excessive caloric intake without a compensatory increase in energy expenditure. In addition, having few meals during the day can be metabolically worse than frequent small meals. This condition is associated with an unsatisfactory response to weight loss therapy and the difficulty in controlling glycaemia in diabetic adolescents.

Binge eating patients are likely to experience other psychiatric symptoms with high negative affect, desire for food and abnormal cognitive control. It is also associated with a significant worsening well-being and worse health outcomes. The psychological risk factors include poor impulse control, adverse childhood experiences, parental depression, and negative comments on the shape.

Binge eating contributes to several metabolic and hormonal changes, such as increased fasting blood glucose and insulin secretion and modulation of diurnal leptin synthesis. Hyperinsulinemia increases the risk of dyslipidaemia, hypertension and, consequently, cardiovascular disease. High levels of insulin can also interfere with the secretion of gonadotrophin and androgens and, consequently, affect the menstrual cycle.

Studies have shown that the gut microbiota of binge eating patients displayed shifts in bacteria, consisting in a lower abundance of *Akkermansia* and *Intestimonas* as well as a higher level of *Bifidobacterium* and *Anaerostipes*. Interestingly, metabolomic analysis revealed that these patients exhibited elevated plasma levels of one food contaminant, bisphenol A bis(2,3-dihydroxypropyl) ether (BADGE.2H(2)O), and a food-derived metabolite, isovalerylcarnitine.

Like bisphenol A, BADGE.2H(2)O is a compound used in the food packaging industry and can be launched in food. A study has shown that BADGE can affect lipid metabolism and disrupt endocrine function. Another recent work showed that BADGE.2H(2)O interrupts testicular function, increasing expression of the nerve growth factor IB (NGFIB), also known as Nur77. Nur77 is a member of the Nur nuclear receptor family of intracellular transcription factors, being involved in cell cycle mediation, inflammation, and apoptosis. It is noteworthy that Nur77 was shown to profoundly influence dopaminergic transmission and to control appetite and sensitivity to leptin.

Isovalerylcarnitine is produced from isovalerate and carnitine by carnitine acetyltransferase and is involved in several biological processes. Isovalerylcarnitine has been shown to be elevated in obesity and cardiovascular disease. Isovalerate, which is produced by fermentation of amino acids (leucine), is harmful to brain function in high doses (isovaleric acidaemia) and is high in the stool of depressive patients. A pre-clinical study demonstrated that isovalerate stimulates the production of γ-aminobutyric acid (GABA) in the brain.

5.3.4. Orthorexia nervosa and bigorexia

Orthorexia nervosa and bigorexia are emerging eating disorders that have been identified recently and are receiving more attention from specialists.

The term orthorexia was derived from the Greek words *ortho* (proper or accurate or correct or right) and *orexia* (appetite or hunger). In 1997, Steven Bratman coined the term orthorexia to define strict adherence to self-imposed dietary rules focusing on the quality and purity of food to such an extent that healthy eating with restricted selected foods (e.g., vegan, vegetarian, and non-organic and/or cooked food) becomes a dominant focus of life. Orthorexia is not a weight loss diet. It is described as a harmful behaviour of eating only pure foods, tendency to exclude foods for fear of

hormones, pesticides, or genetically modified foods, or concerns about animal diseases such as avian flu and mad cow.

To date, no formal diagnosis of orthorexia exists in the Classification of Mental and Behavioural Disorders (ICD-10) nor in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). Although it is not currently a category of DSM-5, orthorexia is believed to have some overlap with other eating disorder diagnostic categories. For example, orthorexia was proposed to exist in a continuum with anorexia with the sharing of symptoms of problematic eating habits and perceptions and was found to be associated with negative impacts on functioning and physical and mental health. The main characteristics of orthorexia are opposed to other eating disorders, such as anorexia and bulimia, who express fear of weight gain, emphasis on the amount of food, prevalent in females and concern about body image disorders.

Although there is some variation between the criteria, the definitions commonly describe the following: an obsessive focus on eating foods that are considered healthy or pure; compulsive behaviour or mental concern related to foods considered unhealthy and emotional distress and fear associated with unhealthy foods and their potential effects on the body and health. In addition, the definitions highlight the need for concern about healthy eating to become harmful in some way (for example, socially, physically, emotionally, or financially) for this to be described as orthorexia and to distinguish this clinically significant problem from exceptionally healthy food behaviours.

In extreme cases, the obsessive-compulsive characteristics of orthorexia become pathological and control a person's life. Due to the obsessive omission of some essential food groups or items, the consequence can be nutritional deficiencies in orthorexic individuals. In addition, it may result in loss of life quality and social relationship.

The consequences on the nutritional status of orthorexic individuals are the same as those that occur due to inadequate nutrition, such as malnutrition, anaemia, hyper or hypovitaminosis, lack of essential nutrients, hypotension, and osteoporosis. In more advanced cases of the disease, the very lack of vitamins can lead to behavioural changes that further accentuate the obsession with healthy foods.

Bigorexia (or muscle dysmorphia), which is a body dysmorphic disorder that mainly affects men, was first defined by Pope and Coll in 1993 as an

obsessive-compulsive disorder, characterised by an obsession with the appearance of the body, fear of not being muscular enough and need for excessive physical exercise. The term bigorexia is included in the scope of "body dysmorphic disorder" in DSM-5 under the main heading "Obsessive-Compulsive Disorders and Related Disorders". However, several researchers have argued about being an eating disorder.

People with bigorexia think they are small and weak, even if they look normal or very muscular. Consequently, they engage in behaviours that aim to achieve the desired lean and muscular physique. Such behaviours are mandatory and may include excessive exercise and a strict diet, overuse of dietary supplements, and sometimes the use of anabolic/androgenic steroids. These obsessions with the body can lead to serious clinical disorders, such as tiredness and fatigue, muscle pain throughout the body, muscle and joint injuries from excessive exercise, insomnia, impaired self-esteem, irritability, high anxiety, depression, suicide and even death.

The inadequate diet (rich in proteins) and the excessive consumption of protein supplements can cause metabolic disorders, especially affecting the kidneys, with increased blood glucose and cholesterol levels. In more severe cases it can lead to kidney or liver failure, vascular problems, and major depression. When abuse of anabolic steroids occurs, cardiovascular diseases, prostate cancer, hair loss and/or decreased testicular tissue, hypogonadism, and gynecomastia, amenorrhoea and irregular menstrual cycles in women can also be present. Clinical tests can reveal anaemia, suppression of thyroid hormones, deficits in sex hormones (oestrogens, progesterone, or testosterone), extremely high cortisol levels, lack of magnesium, zinc, selenium or vitamin D, and accumulation of lactic acid.

Conclusion

Nutritional diseases include a wide range of conditions, such as malnutrition, obesity, and eating disorders. Malnutrition is a pathology that, nowadays is present especially in risk groups (children and the elderly). It is the individual's nutritional status characterised by an insufficient intake of energy and nutrients. This condition causes several changes in biological systems, such as growth and development speed slowdown, changes in the nervous, endocrine, and immune systems and in the function of the pituitary gland, as well as metabolic adaptations like reduced basal metabolism and body temperature, and adjustments of skeletal muscle fibers.

Obesity refers to excess body weight in relation to height. The main mechanism of weight gain is the consumption of more calories than daily energy needs. Obesity in children and adolescents is a growing concern in developed and developing countries. During ageing, subcutaneous fat decreases, while visceral fat accumulates, causing disturbances in the metabolism of glucose and lipids. The whole body's sensitivity to insulin and the intracellular glucose oxidation rate is reduced in the elderly when compared to young adults. Obesity contributes to the development of insulin resistance, which is behind type 2 diabetes mellitus, and represents an important feature of the metabolic syndrome and is commonly observed in the elderly.

Eating disorders usually develop during adolescence or early adulthood. Anorexia nervosa is characterised by extremely low body weight, along with a strong fear of gaining weight and distorted weight perception. Due to prolonged starvation, the catabolism of macromolecules such as fat and proteins is induced, leading to loss of volume and cellular function, and several hormonal changes. Bulimia nervosa is characterised by consuming a large amount of food in one session, followed by an attempt to prevent weight gain by purging what was consumed. Among the biochemical changes assessed, metabolic defects, such as abnormalities in the homeostasis of proteins, glucose, lipids, and enzymes are relatively common. Binge eating disorder is characterised by recurrent episodes of eating large amounts of food over a short period of time. Several metabolic and hormonal changes, such as increased fasting blood glucose and insulin secretion and modulation of diurnal leptin synthesis, are observed. Orthorexia nervosa is described as a harmful behaviour of eating only pure foods. The consequences on the nutritional status are the same as those that occur due to inadequate nutrition, such as malnutrition, anaemia, hyper or hypovitaminosis, lack of essential nutrients, hypotension, and osteoporosis. Bigorexia is characterised by an obsession with the appearance of the body, fear of not being muscular enough and need for excessive physical exercise. The inadequate diet (rich in proteins) and the excessive consumption of protein supplements can cause metabolic disorders, especially affecting the kidneys, with increased blood glucose and cholesterol levels.

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CHAPTER 6

BIOCHEMICAL CHANGES IN INFECTIOUS DISEASES

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List of abbreviations

ACE2: Angiotensin converting enzyme 2
ADA: Adenosine deaminase
AIDS: Acquired immunodeficiency syndrome
ALT: Alanine transaminase
gSG6-P1 peptide: *Anopheles gambiae* salivary gland protein-6 peptide 1
ARDS: Acute respiratory distress syndrome
AST: Aspartate transaminase
ATP: Adenosine triphosphate
1-3-ßDG: 1-3-β-D-glucan
CAGTA: *Candida albicans* germ tube antibody
***cagA* gene:** Cytotoxin associated gene A
***cagE* gene:** Cytotoxin associated gene E
CLI: Circulatory complement-lysis inhibitor
CRP: C-reactive protein
CSF: Colony stimulating factor
DNA: Deoxyribonucleic acid

***dupA* gene:** Duodenal ulcer-promoting gene A
FDA: Food and Drug Administration
GF: Growth factor
gRNA: Genomic RNA
HDL: High-density lipoprotein
HIV: Human immunodeficiency virus
Ig: Immunoglobulin
IL: Interleukin
LDH: Lactate dehydrogenase
LDL: Low-density lipoprotein
MOF: Multiple organ failure
***oipA* gene:** gene coding for an outer inflammatory protein
PCR: Polymerase chain reaction
PCT: Procalcitonin
PfHRP2: *Plasmodium falciparum* histidine-rich protein 2
PG: Pepsinogen
pLDH: *Plasmodium falciparum* lactate dehydrogenase
PTH: Parathyroid hormone or parathormone
RNA: Ribonucleic acid
ROS: Reactive oxygen species
***sabA* gene:** Sialic acid binding adhesin A gene
SARS: Severe acute respiratory syndrome
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
TGF-β: Transforming growth factor β
TNF: Tumour necrosis factor
***vacA* gene:** Vacuolating cytotoxin A gene
WHO: World Health Organisation

Introduction

Infectious diseases, caused by viruses, bacteria, and parasites, are a public health problem that has emerged in recent decades with new specificities. In fact, this problem is recalled by warnings that take many forms, such as the emergence of previously unknown and highly dangerous contagious diseases such as severe acute respiratory syndrome (SARS), fevers caused by Ebola, Marburg and other viruses, the syndrome pandemic of acquired immunodeficiency (AIDS) which, since 1980, has claimed more than 33 million victims. In addition, the global pandemic caused by the possible outbreak of an avian flu epidemic, which brings back to memory the 40

million deaths caused by the Spanish flu at the beginning of the 20th century.

One hundred years after the Spanish flu pandemic and despite scientific progress (medical technological modernization, vaccination, development of the pharmaceutical industry) the world is facing a similar situation. The medical, social, and economic consequences are already considered unambiguous. And at this time the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, which has already caused more than 5 million deaths in the world, including more than 18,000 deaths in Portugal.

Infectious diseases can be acquired through direct contact with the infectious agent or through exposure to contaminated water or food, as well as through the respiratory or sexual route. Often, these diseases can also be transmitted from person to person, being called infectious diseases. Different infectious diseases can alter different biochemical parameters. Very recently, during the development of coronavirus disease - 2019 (COVID-19), changes in several biochemical parameters have been observed, such as in C-reactive protein (CRP) interleukin (IL)-6 and IL-10, lactate dehydrogenase (LDH), amyloid A, albumin, high-density lipoprotein (HDL) cholesterol and procalcitonin (PCT).

This chapter aims to understand and identify the main biochemical changes that occur in infectious diseases, namely in COVID-19, AIDS, tuberculosis, *Helicobacter pylori* (*H. pylori*), *Tinea* and *Candida* infections, toxoplasmosis and malaria. In this sense, the recognition of possible biochemical markers of each disease is highlighted including their use in diagnosis.

6.1. Infectious diseases of viral origin

Viral diseases encompass any illness, whether mild or serious, caused by a virus. They can be transmitted and take the form of an epidemic. There are many different viral illnesses like the common cold, angina, flu, chickenpox, measles, herpes, hepatitis B, AIDS, COVID-19, etc. These diseases are transmitted through saliva, touch, or sexual intercourse. In some cases, there is an incubation period between the infection and the onset of symptoms. This chapter will address two infectious diseases of viral origin, COVID-19, and AIDS.

6.1.1. COVID-19

Definition

COVID-19, caused by a new SARS-CoV-2 beta-coronavirus, has rapidly evolved into a pandemic since it was first reported in December 2019 in Wuhan, China. COVID-19 triggered a global pandemic, affecting more than 260 million people worldwide, surpassing 5 million deaths.

SARS-CoV-2 can cause symptoms such as fever, dry cough, shortness of breath, fatigue, and lymphopenia in infected patients. In more severe cases, infections that cause viral pneumonia can lead to SARS or death. The pathogen can be transmitted from person to person through close contact, respiratory droplets, and aerosols.

Coronavirus is a spherical virus with a single-stranded ribonucleic acid (RNA) genome, belonging to the Coronaviridae family. Its name derives from the ultra-structural crown-like appearance of the spike proteins on the surface of virions. Coronavirus infects humans, as well as many other species of mammals and birds, often causing intestinal, respiratory, neurological, or systemic diseases of varying severity. In terms of genome, coronaviruses are among the largest RNA viruses, being about three times the size of most retroviruses. Coronaviruses are subdivided into four genera: alpha-coronavirus, beta-coronavirus, gamma-coronavirus, and delta-coronavirus.

Coronavirus genomes encode three classes of proteins: structural proteins, accessory proteins, and non-structural proteins. The main proteins are nucleocapsid (N), spike (S), membrane (M) and envelope (E). Protein S is the major viral binding protein and mediates fusion with the host cell membrane and virus entry. Protein N, in close association with viral genomic RNA (gRNA), forms the helical nucleocapsid, which stabilizes by binding to protein M (Fig. 6-1).

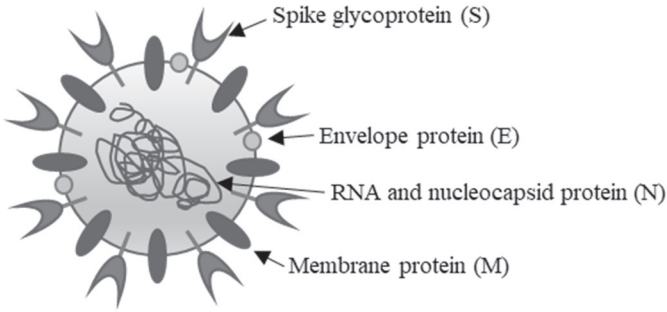

Fig. 6-1. Coronavirus structural proteins.

Biochemical markers

In clinical medicine, biochemical markers used for diagnostic and prognostic measures are usually metabolites or derivatives of metabolites such as enzymes, proteins present in the blood or from a certain tissue or specific deoxyribonucleic acid (DNA) or RNA molecules in the blood, since blood tests are widespread, profitable and the least invasive. Biochemical markers are also classified according to their diagnostic application, namely cardiac markers (cardiac troponin, brain natriuretic peptide), haematological markers, inflammatory markers (CRP, IL-2, IL-6, IL-10), tumour necrosis factor (TNF) and liver markers (aspartate transaminase (AST), alanine transaminase (ALT), LDH, bilirubin, albumin).

Several biochemical alterations have been described in patients with COVID-19. To date, many biochemical markers that reflect the main pathophysiological features of the disease have been identified and associated with the risk of developing serious diseases. Lymphopenia is the main feature of the disease and can be detected early in the infection. In patients with COVID-19, increased levels of several inflammatory biochemical markers have been found, including CRP, which is characterised by the so-called "cytokine storm" associated with an increased risk of severe disease.

Depending on the severity of the disease, the evolution of COVID-19 can be classified into three phases, namely the "early infection phase", the "pulmonary phase" and the "hyperinflammation phase", each characterised by specific biochemical changes (Fig. 6-2).

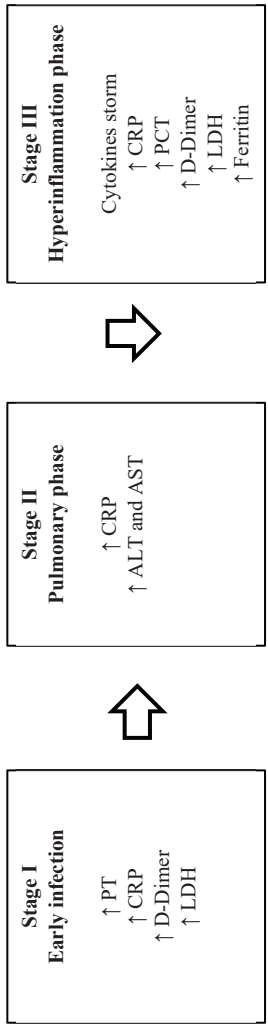

Fig. 6-2. Evolution of the biochemical markers of COVID-19.

The first phase occurs at the time of virus infiltration into the lung parenchyma, where SARS-CoV-2 infects ciliated bronchial epithelial cells through interaction with the angiotensin-2 converting enzyme (ACE2). ACE2 is a monocarboxypeptidase that plays an essential role in regulating the cleavage of various peptides in the renin-angiotensin system. It is highly expressed in lung pneumocytes. At this stage, most patients present non-

specific symptoms, such as dry cough and fever, associated with an initial inflammatory response due to innate immunity, mainly monocytes and macrophages. Lymphocytopenia is a feature of this phase.

The pulmonary phase is characterised by established lung disease (viral pneumonia) associated with localized inflammation in the lung. Biochemical features include lymphopenia and increased transaminases, as well as biological markers of systemic inflammation such as CRP. At this stage, most patients require hospitalisation.

The third phase of COVID-19 is the most severe, being characterised by systemic inflammation, or cytokine storm, leading to acute respiratory distress syndrome (ARDS) and multiple organ failure (MOF). At this stage, patients need to be admitted to an intensive care unit. Several inflammatory biomarkers are significantly increased.

Haematological profile

The haematological profile of patients with severe COVID-19 shows an increase in white blood cell and neutrophil counts, decreased levels of lymphocytes, platelets, eosinophils and haemoglobin. Lymphopenia is the most common laboratory finding in the blood count of patients with COVID-19 from the stage of early infection. Several mechanisms have been proposed to explain the reduction in lymphocyte counts. It was hypothesized that the SARS-CoV-2 virus could directly infect lymphocytes, mainly T cells, causing the depletion of CD4+ and CD8+ cells and, consequently, suppressing the cellular immune response.

The main feature of severe COVID-19 is the host's hyperinflammatory response due to the so-called "cytokine storm", defined as an uncontrolled systemic inflammatory response resulting from the release of large amounts of pro-inflammatory cytokines, a consequence of the activation of natural cell immunity induced by SARS-CoV-2. Cytokines are a group of small proteins secreted by cells of the immune system. They include IL, colony stimulating factor (CSF), interferon (INF), TNF, growth factor (GF) and chemokines.

IL-6

IL-6 is also known as INFβ-2 and B2 cell stimulating factor (BSF-2). It has several biological functions, including the ultimate differentiation of B cells into immunoglobulin-secreting cells. The IL-6 family of cytokines encompasses naturally occurring glycoproteins that contain approximately

170-180 amino acid residues, having four highly conserved cysteine residues that form disulphide bonds.

Increased IL-6 levels result in high mortality in patients infected with COVID-19. It seems likely that the increased pathogenicity of SARS-CoV-2 is related to the more rapid formation of viral replicas and the tendency to affect the lower respiratory tract, resulting in an increased response to severe IL-6-induced respiratory diseases. This IL is an effective biomarker that can be useful in indicating future respiratory failures with better accuracy and helping clinicians to properly categorize patients at an early stage.

CRP

CRP is an acute phase protein mainly synthesized by the liver, which is rapidly released into the bloodstream after the onset of an inflammatory response (severe bacterial or fungal infection, arthritis, autoimmune disease, inflammatory bowel disease, etc.). Several inflammatory mediators including IL-6, IL-1β, transforming growth factor β (TGF-β) or TNF-α, can cause an increase in the levels of this protein. Depending on the severity of the condition, CRP levels can easily rise to several hundred milligrams per liter (mg/L).

This protein is an important biomarker for infectious diseases, which is the case observed in COVID-19. Serum CRP level directly correlates with the rate of progression of several infections. Likewise, in the case of COVID-19 infections, CRP levels increase with symptom severity, making it a useful biomarker for disease progression. CRP levels increase during infections as a defence mechanism because they activate the complementary immune system, increasing phagocytosis to eliminate pathogens. Blood CRP levels directly correlate with disease severity in pneumonia and other respiratory tract infections. The extent of lung damage increases in parallel with peripheral CRP levels in patients, demonstrating that CRP is a very sensitive indicator of disease severity. CRP levels were found to be higher in critically ill patients, even in the early and progressive stages of the infection, when compared to mildly severe patients. Thus, CRP can be very useful in predicting patient status, clinical symptoms, and severity.

PCT

PCT is the precursor of calcitonin being practically undetectable in the plasma of healthy individuals. Although its biological role is not clearly established, PCT may be of clinical interest as an early marker to support the diagnosis of a bacterial infection. The amount of PCT present in the

blood is often related to the severity of the infection and mortality. Several studies have also shown that initial determination and monitoring of PCT can reduce the use and duration of antibiotic therapy, particularly in the context of respiratory infections.

PCT, released by infectious bacterial tissues under the influence of pro-inflammatory cytokines, is a more specific marker of severe bacterial infection than CRP and IL-6. Serum PCT levels in patients with severe bacterial infections are much higher than in patients with simple viral infections or non-specific inflammatory diseases. Studies also suggest that regular monitoring of PCT levels can predict disease progression to a more severe form. This review shows an increase in PCT values associated with an almost 5-fold increased risk of developing serious infections in patients with COVID-19. A substantial increase in PCT would therefore reflect a possible bacterial co-infection in those who develop a severe form of the disease, thus contributing to a more complicated clinical picture. This increase could be supported by increased concentrations of IL-1β, TNF-α and IL-6, markers often present in abundance in cytokine release syndrome.

Ferritin

Ferritin is an iron storage protein in tissues. The determination of serum ferritin concentration is primarily used for the assessment of states of deficiency (iron deficiency anaemia) or overload (hemochromatosis). However, since ferritin is an acute phase protein, its production level can increase very quickly during an inflammatory process (hyperferritinaemia).

Ferritin could be a marker of clinical deterioration, as high levels have been associated with an increased risk of serious complications in patients with COVID-19. In an analysis of the clinical characteristics of 99 patients, 63 of them had serum ferritin well above the normal value with a median value of about 808.7 ng/mL. It was found that people with the severe and very severe forms had increased levels of serum ferritin. In fact, serum ferritin in the very severe COVID-19 group was significantly higher than in the severe COVID-19 group (1006.16 g/L vs 291.13 g/L). The ferritin level is significantly higher in patients who die from COVID-19 (1435 g/L) compared to those who survive (137 g/L).

LDH

LDH is an important enzyme in carbohydrate metabolism, being present in almost all tissues and organs in the human body. An increase in the blood level of this enzyme indicates tissue damage, and it is not possible to

determine the origin of this change. Changes in LDH levels must therefore be supplemented with more biochemical parameters to discover the cause. An elevated LDH level can be seen in several situations, including serious infections. The use of LDH in screening is therefore very rare due to its very low specificity.

In any case, an elevated level of LDH has been reported in over 70% of patients hospitalized with COVID-19. This change may be more common in patients hospitalized with SARS-CoV-2 pneumonia compared to pneumonia of another aetiology. According to a multicentre study in China, patients whose disease progresses to severe pneumonia had higher plasma LDH levels than those with stabilised disease (316.4 ± 86.4 vs. 222.4 ± 73.8). According to the data presented in this study, patients with LDH levels above the normal value had an eight-fold greater risk of disease progression than those with levels close to normal values.

Electrolytic profile

The basic electrolyte profile usually measures sodium (Na^+), potassium (K^+) and chloride (Cl^-), sometimes with the addition of bicarbonate (HCO_3^-). Other elements such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and phosphate (HPO_4^-) can also be quantified if necessary and depending on the clinical situation. Electrolytes play a vital role in the human body and an imbalance can lead to various complications such as dehydration or acidosis/alkalosis. All these elements enter and leave the cells as needed, and the amount depends on food intake and the loss of urine, breath, faeces and sweat. Certain conditions or medications such as diuretics or antihypertensives can also cause an electrolyte imbalance.

Some authors have described changes in electrolyte levels, including Na^+, K^+, Cl^- and Ca^{2+}, in patients with COVID-19. Hyponatraemia, hypokalaemia, and hypocalcaemia have been associated with serious illness. Although the pathophysiological mechanisms underlying these changes are not fully understood, some hypotheses have emerged. The interaction of SARS-CoV-2 with the ACE2 receptor can reduce the expression of the gene encoding this receptor, leading to an increase in angiotensin II, which promotes K+ excretion, resulting in hypokalaemia. Likewise, gastrointestinal involvement, characterised by diarrhoea, could contribute to electrolyte imbalance.

6.1.2. AIDS

Definition

Worldwide, more than 30 million people are infected with the human immunodeficiency virus (HIV), the causative agent of AIDS. HIV infects and destroys cells of the immune system (including a type of lymphocytic cell, CD4).

The first stage of HIV infection can result in a short-lived flu-like illness, but in the second stage, which can last for several years, HIV replicates in the lymph glands (small organs of the immune system scattered throughout the body) without causing any symptoms. However, the immune system eventually becomes so damaged that people infected with HIV begin to succumb to "opportunistic" infections (e.g., bacterial pneumonia) and cancers (in particular, Karposi's sarcoma) that the immune system would normally avoid. AIDS itself is characterised by one or more severe opportunistic infections or cancers (so-called AIDS-related illnesses) and low CD4 cell counts in the blood. HIV infections cannot be cured, but antiretroviral therapy - a combination of powerful antiretroviral drugs - can control the infection. Thus, many HIV-positive people now have significantly longer life expectancy.

HIV can be transmitted through the exchange of various body fluids from infected people, such as blood, breast milk, semen, and vaginal secretions. It can also be transferred from mother to child during pregnancy, childbirth, and breastfeeding. Infections do not occur through casual contact, such as kissing, hugging, shaking hands, sharing personal objects, drinking water or ingested food.

Without treatment, the evolution of HIV in the body can be divided into four phases: primary infection, asymptomatic phase, minor symptomatic phase, and major symptomatic phase.

In primary infection, HIV invades the body from the moment it enters the mucous membranes or bloodstream until complete colonization of lymphoid tissues. Once established, the virus makes copies of itself, releasing new viruses into the bloodstream. This phase, which lasts from a few weeks to a few months, varies greatly from one individual to another, and may go unnoticed or be manifested by numerous flu-like symptoms. The amount of HIV in the blood (viral load) is very high at this stage and, as a result, infected people are at high risk of getting it. CD4 cells drop dramatically under virus attack. The body responds by producing large

amounts of CD8 cells, which in turn produce substances that help neutralize HIV-infected cells, helping to reduce the viral load. The immune system learns to recognize and fight HIV and starts making antibodies against the virus. This seroconversion occurs one to three months after infection.

The asymptomatic phase can last for 5 to 10 years, with a progressive loss of immune system function caused by the virus. CD4 cells drop slowly and steadily.

In the minor symptomatic phase (AIDS phase), if not yet treated, the patient manifests one or more symptoms of HIV infection (fatigue, diarrhoea, swollen lymph nodes, weight loss, night sweats, fever, etc.).

In the main symptomatic phase, the number of immune cells (CD4 T cells) becomes very low, and the body is no longer able to fight off other infections or diseases. The diagnosis of AIDS is established, with the symptoms of the infection being more apparent and constant. Furthermore, opportunistic infections can cause significant health problems. Opportunistic infections are usually not serious, but they become serious in people with very weak immune systems. Examples of opportunistic diseases include candidiasis, pneumonia, tuberculosis, herpes infections, and cancers (including lymphoma and Kaposi's sarcoma).

Biochemical markers

IL-6

IL-6 is a pro-inflammatory cytokine that regulates several physiological processes. It plays a key role in the acute phase response and in the transition from acute to chronic inflammation. Data from several studies suggest that dysregulation in IL-6 production is an important factor in the pathogenesis of chronic inflammatory and autoimmune diseases.

HIV infection induces IL-6 expression and secretion by monocytes and macrophages. Even in virological suppression, individuals with HIV have significantly higher plasma levels of IL-6 than uninfected individuals. IL-6 is strongly associated with all-cause mortality. Discontinuation of antiretroviral therapy may increase the risk of death by increasing levels of IL-6.

D-dimer

D-dimer is a biomarker of fibrin formation and degradation. It can be elevated not only in patients with acute thrombosis, but also in the elderly and in various diseases.

Significantly higher levels of D-dimers were observed in HIV-infected patients with continuous viral replication and lower CD4+ cell counts.

CRP

In humans, CRP is an important acute-phase protein whose concentration can increase over 1000-fold in severe inflammatory conditions. It is a protein composed of five identical, unlinked subunits of 206 amino acid residues, with a molecular weight of ~23 kDa. This protein is increased in HIV-infected individuals.

Cystatin C

Cystatin C is a peptide, member of the superfamily of cysteine protease inhibitors, with a molecular weight of approximately 13 kDa and composed of 122 amino acids. It is produced by almost all human cells and is released into the blood. Cystatin C modulates the inflammatory response, extracellular matrix degradation and phagocytic functions. This protein is also a sensitive indicator of several chronic inflammatory diseases related to oxidative stress and apoptosis. This protein is freely filtered in the renal glomerulus, reabsorbed, and catabolized in the proximal tubules. Furthermore, it is expressed in virtually all organs of the body, such as the adrenal medulla, pancreas, thyroid gland, adenohypophysis, and cortical neurons of the brain. It is used as an alternative measure of kidney function in the general population, more sensitive in detecting mild kidney disease than creatinine-based measures. Cystatin C level is a more sensitive marker of kidney function in chronic disease.

Several risk factors for kidney disease are more common in people with HIV infection. Cystatin C levels are highest in people with HIV infection. Hypertension, hypertriglyceridaemia, inflammation, diabetes, hyperuricaemia, and microalbuminuria are well-described risk factors for reduced kidney function that have been associated with higher cystatin C levels in HIV-infected participants.

6.2. Infectious diseases of bacterial origin

Bacteria are microscopic, unicellular organisms, being among the first known life forms on Earth. There are thousands of different types of bacteria that live in every possible environment, all over the world, such as on land, in the sea and deep in the earth's crust. Some bacteria have even been reported to live on radioactive waste. Many bacteria live on and in the bodies of humans and animals (on the skin and in the airways, mouth, digestive system, genitourinary system) without causing harm. Such bacteria are part of the microbiome of this organism. There are at least as many bacteria in the microbiome as there are cells in the body. Most of the bacteria in the microbiome are useful to humans, for example helping to digest food or preventing the growth of other, more dangerous bacteria.

Only a small number of bacteria cause disease, called pathogenic bacteria. However, sometimes, under certain conditions, the resident bacterial flora can cause illness. Bacteria can cause disease by producing harmful substances (toxins), by invading tissues, or both. Some bacteria can trigger inflammation that can affect the heart, nervous system, kidneys, or digestive tract. Antibiotics may have to be prescribed to effectively fight the bacteria involved.

6.2.1. Tuberculosis

Definition

In 2019, tuberculosis was the most common cause of death from a single infectious pathogen. Worldwide, an estimated 10 million people have developed tuberculosis with 1.2 million deaths among HIV-negative people and an additional 208,000 deaths among people living with HIV. Adults accounted for 88% and children under the age of 15 were 12% of all people with tuberculosis. Most people who developed tuberculosis in 2019 were from Southeast Asia (44%), Africa (25%) and India (18%). In Europe, the percentage is lower (2.5%). In 2020, the COVID-19 pandemic displaced tuberculosis as the leading cause of mortality from infectious diseases worldwide. The main drivers of tuberculosis continue to be malnutrition, poverty, diabetes, smoking and domestic air pollution.

Mycobacterium tuberculosis (*M. tuberculosis*), the most common pathogen causing tuberculosis, is a non-motile, aerobic, rod-shaped bacterium. It is transmitted almost exclusively by droplets. The occurrence of infection depends mainly on the frequency of contact with a person with infectious

pulmonary tuberculosis, the duration and proximity of the contact, the quantity and virulence of the transferred pathogen and the susceptibility of the exposed person.

There are two forms of tuberculosis, pulmonary tuberculosis and extrapulmonary and disseminated tuberculosis. Typical symptoms of pulmonary tuberculosis are fever, night sweats, abnormal fatigue, productive cough, and haemoptysis. In non-immunocompromised adults, the disease progresses slowly. On the other hand, children and immunocompromised individuals can develop fulminant tuberculosis with a sudden onset. The clinical symptoms of extrapulmonary and disseminated tuberculosis can take many forms and are determined by the specific organ(s) involved. Disseminated tuberculosis (which affects two or more organ systems), previously seen almost exclusively in children or immunocompromised individuals, is now increasingly seen in adults with no apparent immunological defects.

The main diagnostic techniques for active tuberculosis are direct microscopic demonstration of the pathogen by culture or nucleic acid amplification (generally procedures based on the polymerase chain reaction (PCR)). The sample to be tested must be obtained before starting treatment.

Biochemical markers

Adenosine deaminase (ADA)

ADA is a purine decomposition enzyme that irreversibly catalyses the deamination of adenosine, resulting in the production of inosine. Its levels in body fluids can be measured quickly and could be an alternative for tuberculosis diagnosis. Several studies have reported the use of ADA in the diagnosis of tuberculosis in other fluids, including meningeal, pleural, and pericardial effusions, suggesting that the increase in ADA activity is related to the intensity of stimulation and the state of lymphocyte maturation, due to the cellular immune response against *M. tuberculosis*.

Calcium and phosphorus

Calcium is one of the body's electrolytes, minerals with an electrical charge when dissolved in body fluids such as blood. Most of the body's calcium is in its uncharged form. About 99% of calcium is stored in bones and can also be found inside cells (especially muscle cells) and in the blood. Calcium is essential for bone and tooth formation, muscle contraction, normal functioning of many enzymes, blood clotting and normal heart rhythm. The

level of calcium in the blood is mainly regulated by two hormones, parathyroid hormone (PTH) and calcitonin. PTH is produced by the four parathyroid glands located behind the thyroid gland in the neck. When blood calcium levels fall, the parathyroids produce more PTH. When blood calcium levels increase, the parathyroids produce less hormone. On the other hand, calcitonin is produced by certain thyroid cells, decreasing the level of calcium in the blood by slightly reducing bone degradation.

Phosphorus is a mineral found mostly (about 80%) in bones and teeth and is closely linked to calcium (calcium phosphate) to ensure adequate ossification. The remainder is associated with lipids to form phospholipids that are part of the composition of cell membranes. Phosphorus also plays an important role in energy production, being an essential component of adenosine triphosphate (ATP, which is the primary source of energy in the human body), and phosphocreatine, which is used to regenerate ATP. Finally, it helps to neutralize acidic compounds resulting from energy metabolism, thus contributing to the body's acid-base balance.

Serum calcium and phosphorus are significantly reduced in pulmonary tuberculosis.

6.2.2. *Helicobacter pylori* infection

Definition

H. pylori is a gram-negative bacterium that infects approximately 4.4 billion people worldwide. However, its prevalence varies between geographic areas and is influenced by several factors. The infection can be acquired through oral or faecal transmission. The pathogen has several mechanisms that increase its ability to move, adhere to and manipulate the gastric microenvironment. In addition, *H. pylori* has a wide variety of genes coding for virulence factors that increase its pathogenicity, including the gene coding for urease, the *cagA* (*cytotoxin associated gene A*) gene that codes for a cytotoxin called cagA protein, the *cagE* (*cytotoxin associated gene E*) gene that codes for a cytotoxin called cagE protein, the *vacA* (*vacuolating cytotoxin A*) gene, the *oipA* (*outer inflammatory protein*) gene that codes for an external inflammatory protein, the *dupA* gene that is associated with the development of duodenal ulcer, and the *sabA* (*sialic acid binding adhesin A*) gene that codes for one of the proteins of the external membrane of *H. pilori*, called sabA protein, an adesin that binds to sialic acid that attaches to an antigen of the human gastric epithelial cell.

Although most *H. pylori*-positive individuals remain asymptomatic, the infection predisposes to the development of various clinical conditions, such as gastric ulcers, gastric adenocarcinomas, and mucosa-associated lymphoid tissue lymphomas. The progression of *H. pylori* infection to gastric ulcers is associated with predominantly antral (lower stomach) gastritis and acid hypersecretion leading to colonization and inflammation of the duodenum, the site of the duodenal ulcer (which accounts for 95% of the disease). The progression of the infection to gastric atrophy and then gastric cancer is usually associated with pangastritis (gastritis of the upper and lower parts of the stomach). It is commonly seen in patients with acid hyposecretion and is more common in people over 50 years of age. Stomach cancer is the third leading cause of cancer death worldwide, with 750,000 deaths each year. The highest rates of mortality from stomach cancer are found in East Asia, Eastern Europe, and countries in Central and South America.

Biochemical markers

Urease

Urease is a cytoplasmic enzyme produced by many bacterial species and is a virulence factor in some bacteria such as *Proteus Mirabilis*, *Staphylococcus aprophyticus* and *H. pylori*. Urease provokes a vigorous immune response in the host and is essential for the good metabolism and virulence of *H. pylori* and will be necessary for the colonization of the gastric mucosa.

Urease hydrolyses urea to ammonia and carbamate. The latter will then decompose to give another molecule of ammonia and CO_2 (Fig. 6-3). The acid tolerance of *H. pylori* is largely dependent on urease activity. The access of urea to the enzyme is limited by the presence of H^+ so that, under acidic conditions, urea can enter the cytoplasmic space and be hydrolysed to CO_2 and ammonia.

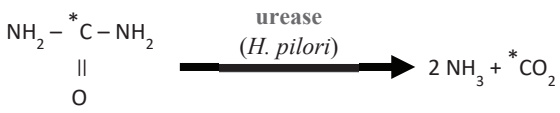

Fig. 6-3. Urease catalysed reaction: Conversion of urea to ammonia and CO_2. The asterisk indicates labelled carbon (see urease detection below).

The main role of urease is to neutralize the acidity in its environment, thus controlling the pH in its host, which is essential for the survival of *H. pylori* in the human gastric mucosa. This action will also allow colonization of the gastric mucosa. In fact, a bacterial species without urease will not be able to colonize its host. Finally, the presence of urease will damage the stomach barrier, since urea catalysis will lead to a large production of ammonia that induces apoptosis of gastric epithelial cells by inflammatory cytokines, damaging the intercellular junctions, with consequent damage of the stomach mucosa. This leads to gastritis, peptic ulcer and gastric cancer.

Thus, the presence of urease allows *H. pylori* to colonize the acidic stomach and serves as a biomarker for the presence of this bacterial strain. Clinical tests for *H. pylori* infection diagnosis are based on urease activity. The rapid urease test involves a biopsy of stomach or mucus that are placed in a container with urea and an indicator of pH change. The presence of urease, and consequently of *H. pilori*, is determined by the detection of pH alteration due to the production of ammonium (Fig. 3-3). A second test is the urea breath assay that measures the change in isotope enrichment of ^{13}C- or $^{14}CO_2$ in breath following oral administration of labelled urea. As shown in fig. 3-3, labelled urea is converted into labelled CO_2, that will be detected in the breath.

Pepsinogen (PG)

PG is a protein synthesized by the main cells of the stomach, being a component of the gastric juice. In its native form, PG has no proteolytic-type enzymatic activity, being the inactive precursor (PGI) of the active enzyme pepsin (PGII). To be converted to the active form, PG must be converted into pepsin in the stomach, being this conversion possible due to the high acidity of the gastric juice. Hydrochloric acid, present in gastric juice, converts PG into (active) pepsin by removing a fragment of the molecule, exposing its active site. The proenzyme thus becomes an active endoprotease enzyme.

Serum PG levels have been identified as a marker of gastric mucosal status, including atrophy and inflammation. The combination of *H. pylori* serology with measurements of serum PGI levels and the PG I/II ratio can be applied to gastric cancer screening.

CagA protein

The cagA protein acts as a highly immunogenic antigen. CagA positive strains are usually more virulent and induce higher expression of cytokines.

This protein can induce the production of inflammatory cytokines such as IL-8, IL-10, and IL-12. The cagA protein is injected in the host cell and induces changes in tyrosine phosphorylation that leads to changes in the signalling transduction pathways, resulting in morphological alterations and cytoskeleton rearrangements.

CagA seropositivity is significantly associated with gastric cancer and duodenal ulcer since this strain induces an intense inflammatory response.

Iron deficiency anaemia

Biologically, iron deficiency anaemia is manifested by a decrease in haemoglobin, microcytosis and hypochromia. Pallor of the skin and mucous membranes, hypoxia and the presence of asthenia are the main clinical signs of iron deficiency anaemia.

H. pylori is now recognized as one of the causes of unexplained iron deficiency anaemia. It has been seen that eradication of *H. pylori* in patients with iron deficiency anaemia was followed by an increase in sideraemia. Moreover, among women with the same diet and iron intake, uninfected women show higher serum ferritin levels than women infected with *H. pylori*.

Vitamin B12 deficiency

Vitamin B12 plays a key role in the rapid renewal of tissues such as skin cells, gastric mucosa, nervous tissue, and hematopoietic cells. The main sources of vitamin B12 are fish and meat, requiring a daily intake of 2.4 μg/day for an adult, according to the Food and Drug Administration (FDA). Secretions of hydrochloric acid in the stomach will hydrolyse the binding of dietary proteins to vitamin B12. This will then be reabsorbed by the enterohepatic cycle at 75%.

Vitamin B12 deficiency remains quite common in the general population, particularly in the elderly. This deficiency can lead to haematological and neurological complications. There are many causes for this deficiency, including *H. pylori* infection. This bacterium can cause vitamin B12 deficiency, including the development of chronic gastritis. This bacterium will cause hypochlorhydria or achlorhydria, leading to a decrease in the absorption capacity of vitamin B12 at the gastric level with poor dissociation of dietary proteins.

6.3. Infectious diseases of fungal origin

Fungi usually cling to moist areas of the body where skin surfaces come into contact: between the toes, in the genital area and under the breasts. Common fungal skin infections are caused by yeasts such as *Candida albicans* (*C. albicans*) or *Malassezia furfur*, or dermatophytes such as *Epidermophyton*, *Microsporum* and *Trichophyton*. These fungi can live on the skin or in the environment. They can be transmitted from an infected person or animal or from the environment.

Many fungi that infect the skin live only in the most superficial and upper part of the epidermis (*stratum corneum*) and do not penetrate any further. Obese people are more likely to suffer from these infections due to excess skin folds, especially when the skin in one skin fold becomes irritated and damaged (intertrigo).

Fungal infections can be devastating in immunocompromised patients. Therefore, early diagnosis followed by rapid initiation of appropriate antifungal therapy is fundamental to significantly improve patient survival.

However, the progression of fungal infections is fast and are difficult to diagnose, especially at early stages. Diagnosis tests can include culture-based approaches for the detection of fungal species and identification of resistances, and non-cultural diagnostic tests. These last ones have the advantages of faster detection and higher sensitivity and are based on the detection of a biochemical marker, such as *Aspergillus* immunoglobulin (Ig)G antibody in chronic pulmonary aspergillosis and galactomannan antigen testing.

6.3.1. *Tinea* infection

Definition

Skin infections are caused by dermatophytes and are classified according to the affected site. The most common in prepubertal children are *Tinea corporis* and *Tinea capitis*, while adolescents and adults are more likely to develop infections with *Tinea cruris*, *Tinea pedis* and *Tinea unguium* (onychomycosis).

Ringworm is a common infection of the skin and nails that is caused by a fungus. It's called a "worm" because it can cause a circular, red, itchy rash on the skin. About 40 different species of fungi can cause ringworms.

Biochemical markers

CRP

CRP is a protein of the acute phase of inflammation, mainly produced by hepatocytes as part of an inflammatory response. In addition to serving as a marker of systemic inflammation, CRP plays an important role in the inflammatory process, being involved in opsonization and activation of the complement system in response to IL-6 secretion. Some studies confirm that chronic ringworm has a considerable systemic inflammatory component, being directly related to the overall inflammatory burden of the skin.

Lipid profile

In ringworm, the serum lipid profile (total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides) is also affected, and the levels of these different molecules are abnormally low. Dermatological disorders are associated with dyslipidaemias. Some of the dermatological therapies are known to predispose to lipid abnormalities.

6.3.2. *Candida* infection

Definition

Candida species are yeasts that belong to our natural microflora, being found in the gastrointestinal tract, the mouth, and the vagina. However, overgrowth and infections can happen, being the most prevalent cause of fungal infections in humans.

There are several types of *Candida* infections, including urinary tract infections. In fact, this is the most common cause of fungal urinary infections and can affect only the lower portion of the urinary tract or can even ascend up to the kidneys.

This yeast is also the most common cause of genital yeast infections. Usually, the level of *Candida* present in the genital area is kept under control by the bacterial species *Lactobacillus*. However, if the level of this bacteria is somehow disrupted, an overgrowth of *Candida* can occur and cause infection leading to symptoms like burning or painful feeling in or around the vagina, among others. *Candida* species can also infect male genitals, causing an itchy or burning rash around the head of the penis.

Mucocutaneous candidiasis is also caused by infection of the skin and mucous membranes by *Candida* species, being the most frequent agent *C. albicans*. This leads to red rash or blister-like lesions in the affected area.

Although *C. albicans* belongs to the normal microflora of the mouth, its increased growth can lead to infection, causing oral thrush. This infection may not be limited to just the mouth and can spread to the tonsils and the back of the throat and in severe cases can even reach the oesophagus. If not treated, this infection can lead to a systemic infection, especially in patients with compromised immune system.

Less frequent but very serious infections can result from the entrance of *C. albicans* into the bloodstream, possibly causing severe problems in blood (candidemia). Symptoms resemble the ones observed from bacterial sepsis, such as fever, kidney failure and shock.

The presence of *Candida* species in blood can also lead to severe problems in other organs. For instance, if the infection reaches the heart, can lead to endocarditis (infection of the inner lining of the heart, including chambers and valves). If *Candida* travels through the blood and reach the spinal cord, can provoke fungal meningitis that is evidenced by headaches, stiff neck, fever, nausea, and vomiting. Endophthalmitis is an inflammation of the eye caused by fungus that can lead to vision loss. The most common agent of this condition is *C. albicans*. *Candida* peritonitis is an intra-abdominal candidiasis, an inflammation of the lining of the inner abdomen. At last, *Candida* species can also be responsible for osteomyelitis or arthritis, although bacterial infections are more frequently the causing agents of these conditions.

Biochemical markers

Mannan, anti-mannan and C. albicans *germ tube antibody (CAGTA)*

These are the nonculture diagnostic tests first developed for diagnosis of invasive candidiasis. These methods are focused on the detection of *Candida* antigens or anti-*Candida* antibodies.

CAGTA is a technique developed for the detection of antibodies against *C. albicans* antigens in the mycelial phase when this organism is invading tissues. It was found that it can also be used for the detection of other *Candida* species. This method allows the quantification of the produced antibodies and is used for the diagnosis of invasive candidiasis.

The major problem with these assays is that sensitivity may be decreased in immunocompromised individuals. Other concerns are the time of the assay needed to have detectable responses and a positive result does not always allow to distinguish an acute from a past infection.

In general, measurement of serum IgG usually performs better than IgM. However, the diagnostic value of this method can be improved if combined with other biomarkers.

1-3-β-D-glucan (1-3-ßDG)

1-3-ßDG is an important constituent of the cell wall of *Candida* species and of most pathogenic fungi. Several assays have been developed for the diagnosis of invasive fungal infections in serum. These assays do not directly measure the levels of 1-3-ßDG. Instead, they are indirect colorimetric or turbidimetric assays. The methods involve the quantification of the rate of activation of a horseshoe crab coagulation cascade that is activated after binding to 1-3-ßDG.

1-3-ßDG data are complicated to interpret due to several factors such as the heterogeneity of patient and control populations, and the types of *Candida* species, among others. Moreover, the major problem with this assay is the frequency of false positive results.

6.4. Infectious diseases of parasitic origin

A parasite is an organism that lives on or in another organism (the host), benefiting from the host, for example, by obtaining nutrients. Parasitic infections can be caused by protozoa, which are made up of only one cell, and worms (helminths), which are larger and made up of many cells, containing internal organs.

Parasites normally enter the body through the mouth or skin. The orally acquired (mouth) parasites are ingested and can remain in the intestine or can also cross the intestinal wall and invade other organs. Often, the parasites enter the mouth through faecal-oral transmission. Some parasites can enter directly through the skin. Still others are transmitted through insect bites.

6.4.1. Toxoplasmosis

Definition

Toxoplasmosis is a widespread cosmopolitan infectious disease of humans and animals, caused by the parasite *Toxoplasma gondii* (*T. gondii*). Although it is most often benign in an immunocompetent person, a primary *T. gondii* infection can be much more severe in cases of immunosuppression or when it is congenital. In pregnant women, this infection is serious since it can affect the development of the foetus and later the young child.

T. gondii is a parasite with obligate intracellular development, belonging to the order Coccidia, phylum Apicomplexa. This is the only currently known species of the Toxoplasma genus and was discovered for the first time by Nicolle and Manceaux at the Pasteur Institute in Tunis in 1908, in the tissues of a small wild rodent *Ctenodactylus gondii*. *T. gondii* also derives its name from the Greek word "toxon" which means "bow", which reminds its shape. *T. gondii* can parasitize many hosts, being the cat its definitive host and all homothermic animals as potential intermediate hosts.

In a healthy person, toxoplasmosis causes few symptoms, which are like flu: mild fever, muscle pain, fatigue, and swollen glands. These symptoms resolve spontaneously. In immunocompromised people, toxoplasmosis leads to severe neurological symptoms such as seizures and sometimes pneumonia. In the foetus, this infection is more serious when it occurs early in pregnancy and can lead to serious developmental abnormalities and even miscarriages. When the infection occurs later in pregnancy, disturbances in eye development may occur. In addition, the newborn may develop jaundice, enlarged spleen and liver, or seizures.

Biochemical markers

CRP

CRP is an essential component of the non-specific immune response, which increases during infection and inflammation. Cases of acute toxoplasmosis show a significant increase in plasma CRP.

IgM and IgG

The primary infection of toxoplasmosis results in the appearance of specific anti-toxoplasma antibodies in the serum, which is called seroconversion. It is the detection of this seroconversion, defined by the appearance of specific

IgM and then IgG antibodies, that will allow the detection of a primary toxoplasma infection. It is essential to master the kinetics of these antibodies to correctly interpret the results of the serological tests performed. 5 successive serological phases can be distinguished:

Phase 1: Absence of IgM and IgG. This is the latency phase between contamination and the onset of the humoral response. It lasts approximately 8 to 10 days.

Phase 2: Presence of IgM and absence of IgG. This is the very early stage of the antibody response. IgM synthesis always occurs before IgG synthesis. This phase can last from a few days to several weeks because the delay in IgG synthesis compared to IgM is very variable.

Phase 3: Presence of IgM, appearance and increase of IgG. Infection with *T. gondii* is confirmed by the appearance of IgG and an increase in its titer. The maximum level of IgG is reached within 2-6 months after infection. The observation of a significant increase in IgG between two successive serological tests, associated with the presence of IgM, confirms the recent nature of the infection.

Phase 4: Presence of IgM and stabilization of IgG. IgM is most often present during the first 6 months of infection but is often found up to more than a year later. IgG levels peak at the beginning of this phase, remaining on a plateau for a variable period, usually several months, before slowly declining.

Phase 5: Absence of IgM and presence of IgG. This stage corresponds to a so-called "old" infection that dates back at least 6 months. The IgM disappeared. IgG persists at a low level for at least 10 years or even for life. The title is normally stable. In case of toxoplasma reactivation, an increase in IgG is observed.

IgA

The short duration of IgA antibody synthesis is more specific for the first few months of infection and can sometimes differentiate between an acute infection and a chronic infection. But its presence is inconsistent and cases of persistence beyond a year have been observed. Therefore, IgA tests are not systematic in pregnant women. On the other hand, IgA has the particularity (like IgM) of not crossing the placental barrier. Its presence in the blood of newborns can thus allow the diagnosis of a congenital infection at birth.

6.4.2. Malaria

Definition

The *Plasmodium* parasite is the causing agent of malaria. This parasite can be transferred to humans through bites of infected mosquitoes. Once a mosquito bites an infected person, becomes infected and can spread the parasite to other individuals. After being bitten, the parasite enters the person bloodstream and goes to the liver, where the infection develops. After that, the parasite re-enters the bloodstream and invades red blood cells. This infection agent multiplies in red blood cells and when these cells burst (every 48-72 hours), release more parasites into the bloodstream.

The *Plasmodium* parasite can never be transmitted from person to person. Very rarely, the spread of malaria can occur through blood transfusion or by sharing needles.

From the several types of *Plasmodium* parasites, only five can provoke the development of malaria in humans. The most common one, mainly found in Africa, is *Plasmodium falciparum* (*P. falciparum*) which is responsible for most malaria deaths all over the world. This parasite species can lead to severe malaria syndromes in children younger than 5 years of age. Children can develop severe syndromes including severe malaria complications, such as cerebral malaria, severe malaria anaemia or have a mild uncomplicated infection. Cerebral malaria is life-threatening being manifested as complex pathophysiological changes, such as obstruction of cerebral microvasculature, neurological disruption due to hypoxia and higher permeability of the blood-brain barrier.

In Asia and South America, it is possible to find *Plasmodium vivax* that causes a mild form of malaria. However, this species can stay in human liver for 3 years, being responsible for relapses. Quite uncommon is *Plasmodium ovale*, mainly found in West Africa. This species can stay in the liver for several years without causing any symptoms. Finally, *Plasmodium malariae* and *Plasmodium knowlesi* are very rare and are found respectively in Africa and in southeast Asia.

Detection of parasite-related, mosquito-related or host-related biomarkers can improve diagnosis, can allow the distinction between symptomatic/asymptomatic, or uncomplicated/severe infections. This may provide better prognostic indicators and help in treatment guidance.

Biochemical markers

P. falciparum *histidine-rich protein 2 (PfHRP2) antigens*

Most rapid diagnostic tests for malaria, used worldwide, involve the detection of histidine-rich protein 2 (PfHRP2), a bioproduct of *P. falciparum*. PfHRP2 is a glycoprotein produced during the asexual lifecycle and in early sexual stages of *P. falciparum*. This protein is expressed on the surface of infected red blood cells, being released into the peripheral bloodstream, during schizogony.

Since during part of the asexual lifecycle, it is not possible to detect this parasite by microscopy, the detection of PfHRP2 can help in a more accurate diagnosis and in determining the prognosis of severe malaria cases. This method is also very helpful in diagnosis of malaria during pregnancy since *P. falciparum* infections can be undetectable due to their specific adhesion to chondroitin sulphate A in the intervillous spaces of the placenta.

It has been suggested that the detection of PfHRP2 alone has limited clinical specificity for the diagnosis of malaria in regions of high transmission, since this protein remains in the bloodstream even after the clearance of the parasite.

P. falciparum *lactate dehydrogenase (pLDH) antigens*

Rapid diagnostic tests for malaria also involve the detection of LDH present in all *Plasmodium* species (pLDH) infecting humans.

P. falciparum is a microaerophilic organism. As mentioned before, the parasite lifecycle involves two different hosts, the mosquito and the human being. During its lifecycle, this parasite suffers cellular morphological changes and is under variations of oxygen pressure. The transfer from one to the other host implies metabolic adaptations and consequent changes in ultrastructural and physiological organisation of its mitochondria. Oxygen pressure varies from up to 13% in human lungs to 21% in mosquito salivary glands. Metabolic adaptations, required for parasite survival, involve, in the presence of oxygen, the production of ATP through aerobic respiration and glycolysis. The microaerophilic metabolism is probably an adaptation to prevent oxidative stress. LDH catalyses the reversible conversion of pyruvate into lactic acid, allowing the completion of anaerobic ATP

production. This is required for parasite survival since it avoids production of reactive oxygen species (ROS).

In contrast with PfHRP2, pLDH does not persist in circulation after clearance of infection. For this reason, this is a better biomarker of acute and current infection. This enzyme may also be a suitable predictor of treatment failure since its disappearance from blood tracks closely the clearance of the parasite. However, this test has lower sensitivity when compared with PfHRP2 quantification.

Circulatory complement-lysis inhibitor (CLI)

CLI suffers strong blood depletion in cases of severe malaria, such as in children having cerebral malaria. This marker allows to differentiate cases of cerebral malaria from other clinical manifestations of malaria or from non-malaria encephalopathy-like syndromes. CLI binds to complement complexes and leads to complement inhibition. In severe malaria, complement deposition on red blood cells contributes to acute clearance of infected and uninfected erythrocytes. This finding supports the depletion of CLI in blood, in cases of severe malaria.

CLI is found in most body fluids and mammalian tissues and binds to IgG, heparin, bacteria, leptin and beta-amyloid. CLI is important in neuronal protection and cytoprotection. Being a secreted chaperone, CLI is also involved in cell clustering and aggregation, being also a regulator of inflammatory and complement pathways. These functions are probably involved in the pathophysiology of cerebral malaria. So, a possible role of CLI in this pathological condition could be at the blood-brain barrier in places having membrane destruction due to the adhesion of infected erythrocytes.

Studies observed induction of CLI expression by TGF-β and TNF, and an inverse correlation of CLI levels and IL-6, IL-8 and IL-10, at onset of cerebral malaria. These findings support the possible association of low CLI plasma levels and the development of inflammatory conditions. These are strong evidences of the protective role of CLI in inflammation.

Anopheles gambiae *salivary gland protein-6 peptide 1 (gSG6-P1 peptide)*

The identification of proteins present in the mosquito saliva can be an adequate method to measure human exposure to the transmitting vector of malaria. This is the case of gSG6-P1 peptide produced in the salivary glands of *Anopheles*, that has been used as a pertinent biomarker of its bites. This

molecule is highly conserved among *Anopheles* mosquitoes and, even in cases of low exposure to mosquito bites, it is observed a human IgG response to the presence of the gSG6-P1 peptide.

Thus, IgG response to the presence of gSG6-P1 peptide can be used for malaria control through the evaluation of the level of heterogeneity of human exposure to *Anopheles* bites and can also be used to evaluate the efficacy of mosquito control strategies.

Conclusion

With an infectious disease installed, there are several biochemical changes that can be observed. These changes are neither the same nor common to all individuals, and it should also be noted that in many they do not occur. But, in general, it is possible to measure and evaluate these changes.

Currently, the use of biochemical markers extends to several areas of medicine. In infectious diseases, biochemical markers are an integral part of the diagnosis and monitorisation of the treatment, allowing to determine the progression of the disease or the response of a given patient to a drug. The evolution in this area and advances towards increasing knowledge about the biochemical changes of each disease have been promising.

This chapter allowed to recognize the importance of different biochemical markers of each infectious disease. Thus, the biochemical markers mostly used for the diagnosis of infectious diseases and observed changes in their profile were identified. CRP, IL-6, PCT, LDH, electrolytes, urease and Ig integrate some of the most important ones.

Biochemical markers, due to their potentialities, should be used in clinical practice and be an integral part of the diagnosis of various pathologies.

Due to their fundamental role in diagnosis, future research should address the identification of new biochemical markers in disease.

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