Universidade de Aveiro Secção Autónoma das Ciências da Saúde
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Biomarcadores Inflamatórios na Doença de Alzheimer

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Ana Gabriela Henriques, Professora auxiliar convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro.

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o júri

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palavras-chave

Doença de Alzheimer, Neuroinflamação, Citocinas, Quimiocinas, Proteína Percursora de Amilóide.

resumo

A doença de Alzheimer (DA) é o tipo de demência mais comum. Histopatologicamente é caracterizada pela presença de tranças neurofibrilares intracelulares (TNF) e de placas senis extracelulares (PS), as quais estão rodeadas pela microglia e por astrócitos. A neuroinflamação tem sido associada com várias doenças neurodegenerativas. Na DA o processo inflamatório, desencadeado pelo aumento da produção e agregação do péptido Aβ, desempenha um papel fundamental na patogénese da doença. Nas fases inicias, a inflamação possui um papel benéfico na patologia, uma vez que tem sido proposto que a microglia e os astrócitos quando ativados estão envolvidos na remoção de β-amilóide (Aβ). No entanto, a ativação crónica da microglia conduz à produção excessiva de componentes inflamatórios, incluindo citocinas. Isto provoca alterações na expressão e processamento da proteína percursora de amilóide (PPA), estimulando o aumento da produção e acumulação de Aβ, fosforilação anormal da proteína Tau e, consequentemente, efeitos neurotóxicos e perda de neurónios. Uma vez que a neuroinflamação crónica é uma característica da DA, proteínas inflamatórias poderão constituir potenciais candidatos a biomarcadores que auxiliem no diagnóstico clínico desta doença. Desta forma, o principal objectivo deste trabalho foi identificar biomarcadores inflamatórios para a DA através da técnica de citometria de fluxo. Para tal, foram analisadas amostras de plasma de doentes que foram, previamente, examinados por testes de avaliação cognitiva, *clinical dementia rating* (CDR) e *mini mental* (MM). Os sujeitos foram divididos em três grupos distintos, o grupo controlo (CDR-/MM-) e dois grupos de pacientes, CDR+/MM- e CDR+/MM+. O primeiro grupo de pacientes pode conter indivíduos com ligeiras alterações cognitivas (MCI) e o segundo inclui 5 pacientes clinicamente diagnosticados para DA. A análise dos dados revelou diferenças nos níveis de proteínas inflamatórias de ambos os grupos de doentes (CDR+/MM- e CDR+/MM+) em comparação com os indivíduos saudáveis (CDR-/MM-). Os níveis plasmáticos de interleucina-8 (IL-8) foram estatisticamente deferentes ($p<0,05$) do grupo controlo. Correlação significativa entre as concentrações de IL-8 e os estados de CDR foi identificada. Adicionalmente, foram observadas correlações entre MCP-1 e IL-8 e a IL-6. Em conjunto, estes resultados sugerem que a IL-8 poderá ser um potencial biomarcador não só para a DA mas também para o diagnóstico precoce de demência.

keywords Alzheimer's Disease, Neuroinflammation, Cytokines, Chemokines, Amyloid Precursor Protein.

abstract Alzheimer's disease (AD) is the most common form of dementia. Histopathologically it is characterized by the presence of two major hallmarks, the intracellular neurofibrillary tangles (NFT) and the extracellular senile plaques (SP), which are surrounded by activated astrocytes and microglia. Neuroinflammation has been associated with some neurodegenerative diseases. In AD the inflammatory process, prompted by increased Aβ production and aggregation, was reported to have a fundamental role in disease pathogenesis. In early stages the inflammation could have a beneficial role in the pathology, since it has been proposed that the microglia and astrocytes activated could be involved in (amyloid β) Aβ clearance. Nevertheless, the chronic activation of the microglia leads to excessive production of the inflammatory components, including cytokines. It promotes alterations in amyloid precursor protein (APP) expression and processing, stimulating the increase of Aβ accumulation, abnormal Tau phosphorylation and, consequently, neurotoxic effects, irreversible damage and loss of neurons. Since chronic neuroinflammation is a feature of AD, inflammatory proteins may constitute potential biomarkers candidates to assist clinical diagnosis of this dementia. Thus, the main aim of this study was to identify putative inflammatory biomarkers for AD by flow citometry analysis. For plasma samples of individuals examined by clinical dementia rating (CDR) and mini mental (MM) diagnostic tests were used. Subjects were subdivided in 3 distinct groups, a control group (CDR-/MM-) and two patient groups, CDR+/MM- and CRD+/MM+, the former may include mild cognitive impairment (MCI) patients and the latest group included 5 patients clinical diagnosed as AD. Data analysis revealed differences in the inflammatory proteins levels of both patients groups (CDR+/MM- and CDR+/MM+) in comparison to healthy individuals (CDR-/MM-). Interleukin-8 (IL-8) plasma levels were statistically different ($P<0,05$) from control group. Significant correlation between IL-8 concentrations and the CDR stages was also identified. Additionally, correlations of monocyte chemoattractant protein-1 (MCP-1) with both IL-8 and IL-6 were observed. Taken together these findings suggested that IL-8 could be a potential biomarker not only for AD but also for diagnosis of initial stages of dementia.

Abbreviations:

AD	Alzheimer Disease
AICD	APP Intracellular Domain
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
Aβ	Amyloid β peptide
BACE	Beta-site APP Cleaving Enzyme
BBB	Blood Brain Barrier
BCA	Bicinchonic Acid
BCSFB	Blood-Cerebrospinal Fluid Barrier
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
CCR	CC Receptors
CD	Cluster of Differentiation
CdK	Cyclin-dependent Kinase
CDR	Clinical Dementia Rating
CI	Confidence Interval
COX	Cyclooxygenase
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
CXCR	CXC Receptors
C1 – C9	Complement Factors
DAMP	Damage-Associated Molecular Patterns
DLB	Dementia with Lewy Bodies
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EOAD	Early-Onset AD

ERL	Glutamate-Leucine-Arginine Motif
ERK	Extracellular Signal-Regulated Kinase
FAD	Familial Alzheimer's Disease
FBS	Fetal Bovine Serum
FTD	Frontotemporal Dementia
GDNF	Glial-Derived Neutrophic Factor
GDS	Geriatric Depression Scale
GSK	Glycogen Synthase Kinase
H_0	Null Hypothesis
HD	Huntington Disease
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
INF	Interferon
iNOS	inducible Nitric Oxide
LOAD	Late-Onset AD
MAPK	Mitogen-Activated Protein Kinase
MCI	Mild Cognitive Impairment
MCSF	Macrophage Colony-Stimulating Factor
MCP-1	Monocyte Chemoattractant Protein 1
MEM	Minimal Essential Medium
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MM	Mini Mental
MRI	Magnetic Resonance Imaging
NFkB	Nuclear Factor-kappa B
NFT	Neurofibrillary Tangles
NO	Nitric Oxide
NOD	Nucleotide-Oligomerization Binding Domain
NSAID	Non Steroids Anti-Inflammatory Drugs
PAMP	Pathogen-Associated Molecular Patterns

PBS	Phosphatase Buffer Salt
PET	Positron Emission Tomography
PHF	Paired Helical Filaments
PGE2	Prostaglandin E2
PRR	Pattern Recognition Receptors
PSEN1	Presenilin 1
PSEN2	Presenilin 2
P-Tau	Phospho-Tau
PE	Phycoerythrin
RAGE	Receptors for Advanced Glycosylation End Products
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RXR	Retinoid X Receptors
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SP	Senile Plaques
SPECT	Single Photon Emission Computerized Tomography
TLR	Toll-Like Receptors
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
T-Tau	Total-Tau

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I. Introduction

1.1. Concept of inflammation

Our body is constantly exposed to several exogenous and endogenous agents, which can disturb its normal function and, consequently be harmful for us. The organism has various defense mechanisms to fight and remove or eliminate these injurious components, in order to maintain the homeostasis and ensure function of all organs and tissues.

The inflammatory process is a defense mechanism extremely important under pathological conditions, consisting in a physiological immune response that occurs in vascularized tissues (Ratner et al. 1996), against a lesion or damage caused by physical agents, chemicals or microorganisms. Several inflammatory cells are involved in this process, such as lymphocytes, neutrophils, macrophages, among others. After damage, many signaling cascades are activated to prevent severe consequences. Acute inflammation is the first process and can progress to chronic inflammation if the inflammatory stimulus persists for a long period, previously impacting on health (Almeida et al. 2012; Schmidt-bleek et al. 2014).

1.1.1. Acute inflammation

Acute inflammation consists in a quick response (from minutes to days) to a foreign agent and is responsible to trigger defense mediators of the organism to the injury site. Acute inflammation can be divided in two phases: vascular and cellular. The acute vascular response results from vasodilation and increased capillary permeability due to vascular endothelium alterations. Consequently, blood flow increases causing redness, exudation of fluid and plasmatic proteins (edema) and migration of leukocytes (predominantly neutrophils) into the damaged tissues, named of Exudation Process (Kumar et al. 2009).

When injury is severe to the tissues, or if infection occurs, the acute cellular response takes place over the next few hours. This phase is characterized by a sequence of events that begin with the appearance of monocytes and with an increasing amount of neutrophils into extravascular tissue (Brown and Badylak 2013). Due to the presence of adhesion molecules, such as selectins, immunoglobulins, integrins and glycoproteins, on the surface of the neutrophils and in the endothelial surfaces (Ratner et al. 1996; Kumar et al. 2009), attachment of these cells within the blood vessels occurs, followed by cell crossing through the endothelium. The first event is a process called margination, and the second called diapedesis. Then, the leukocytes migrate to the injured site along a chemotactic gradient, which is generated by chemokines (Section 1.3.1.2.) in order to ensure that leukocytes are recruited to the tissues where the stimuli is present. This process is defined as chemotaxis and occurs by binding of granulocytes, monocytes and

lymphocytes (minor percentage) to the leukocytes surface receptors in response to chemotatics stimulus (Kumar et al. 2009).

Activated neutrophils and macrophages can lead to death of the injurious microorganism by two different processes, phagocytosis and enzymes release. Phagocytosis is a three-step process in which the injurious agent undergoes recognition and neutrophil attachment, engulfment, and killing or degradation (Ratner et al. 1996; Kumar et al. 2009). Nonetheless, during chemotaxis and phagocytosis, activated leukocytes can release toxic metabolites and proteases which can be responsible for the tissue lesion (Kumar et al. 2009). Figure 1 shows the multistep process of leukocyte migration through blood vessels.

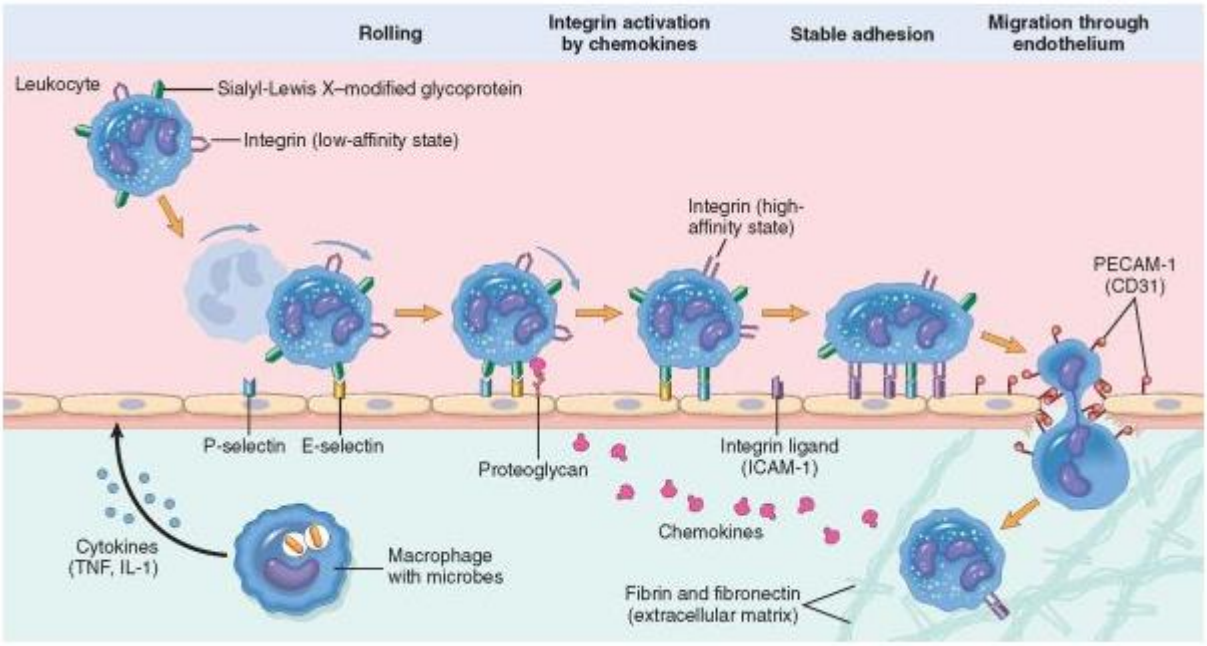


Figure 1 – Migration process of leukocytes through the blood vessels. First, leukocytes (neutrophils) undergo roll, becoming activated and adhering to the endothelium. Then transmigrate across the endothelium, pierce the basement membrane, and migrate toward chemoattractants emanating from the source of injury. Different molecules play an important role in different steps of this process: selectins are involved in roll; chemokines are related to neutrophils activation to increase integrins avidity; integrins are associated to firm adhesion; and CD31 (PECAM-1) in transmigration. Neutrophils express low levels of L-selectin and they bind to the endothelial cells through P- and E-selectins. ICAM-1, intercellular adhesion molecule 1; TNF, tumor necrosis factor (taken from Kumar et al. 2009).

Acute inflammation can be triggered by infections (bacterial, viral, fungal, parasitic) and microbial toxins; by tissues necrosis from any cause such as ischemia, trauma and physical and chemical agents (irradiation, environmental substances); foreign bodies, for example spliters, dirt and sutures; immunological reactions or hypersensitivity reactions (Kumar et al. 2009).

1.1.2. Chronic inflammation

Chronic inflammation has a prolonged duration (from weeks to months) and may follow acute inflammation when the damage is sufficiently severe and/or if there is a prolonged exposure to the initial inflammatory stimuli (Figure 2) (Ratner et al. 1996; Kumar et al. 2009). It can be initiated by persistent infections by microorganisms, such as mycobacteria, some viruses, fungi and parasites; immune-mediated inflammatory diseases (autoimmune diseases); and prolonged exposure to potentially toxic agents, which can be either exogenous or endogenous. Alternatively, it may begin insidiously without any manifestations of an acute reaction (Figure 2), causing tissue damage in some of the most common human diseases, including rheumatoid arthritis, atherosclerosis, pulmonary fibrosis and, more recently, it has also been related to Alzheimer disease (AD) (Kumar et al. 2009).

Chronic inflammation is characterized by the presence and infiltration of mononuclear cells such as macrophages (Brown and Badylak 2013), lymphocytes and plasma cells (Ratner et al. 1996); tissue destruction induced mainly by inflammatory cells; and, ultimately, reparation of the damaged tissue. The latter step involves connective tissue replacement with proliferation of small blood vessels, a process called angiogenesis, and fibrosis of the tissue (Kumar et al. 2009). Macrophages are responsible to produce and secrete a lot of biologically active products, including pro- and anti-inflammatory cytokines (Brown and Badylak 2013), complement components, chemotactic factors, neutral proteases, arachidonic acid metabolites, reactive oxygen species, coagulation factors and growth-promoting factors (Ratner et al. 1996). In addition, macrophages are involved in microbial killing, cleaning up cellular and tissue debris, and they also seem to be very important in tissues remodeling. For all of these reasons, macrophages are considered the most important cells in chronic inflammation.

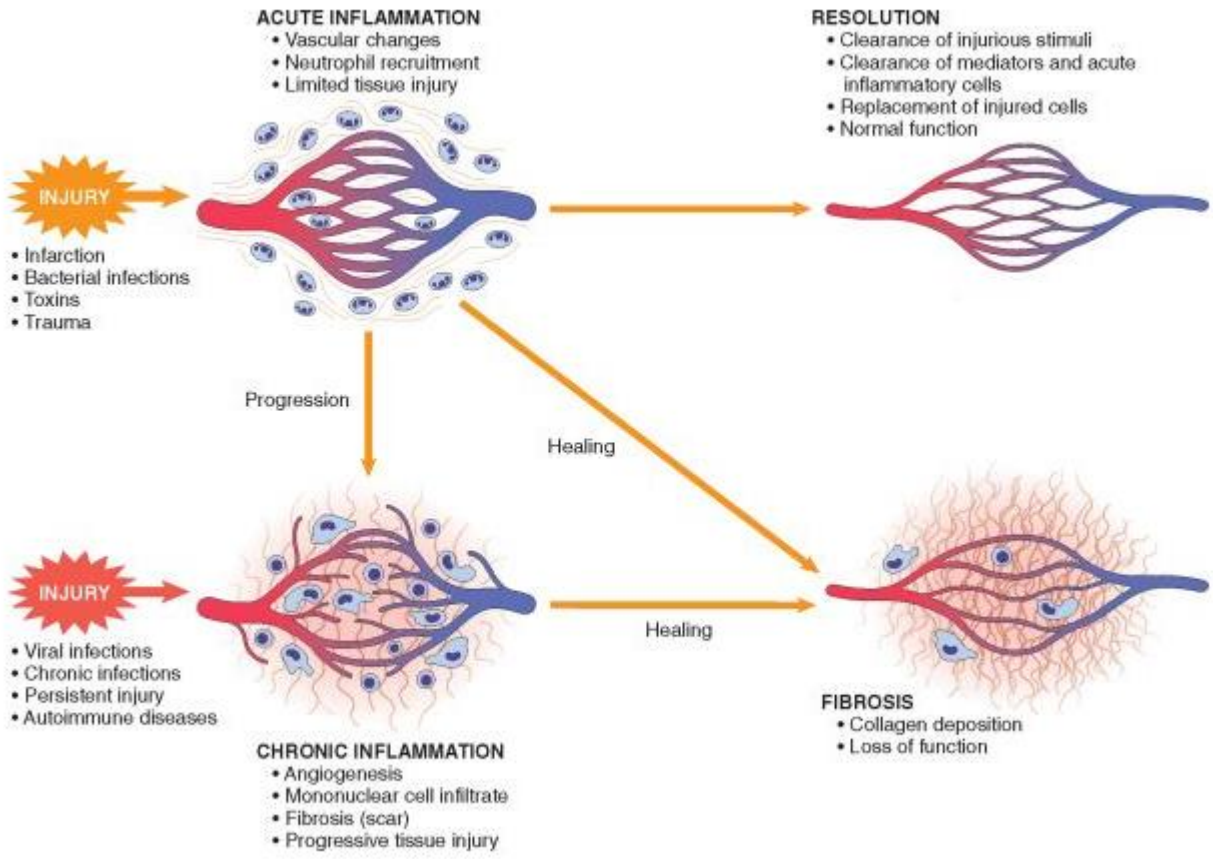


Figure 2 – Chronic inflammation causes: acute inflammation or injury. If acute inflammation persists it can progress to a chronic inflammatory process. The components involved in each reaction are described in the scheme (adapted from Kumar et al. 2009).

In summary, the main features of the inflammatory response include: vasodilation, i.e. widening of the blood vessels in order to increase the blood flow to the infected area; increased vascular permeability, which allows diffusible components to enter the site; cellular infiltration by chemotaxis, or the directed movement of inflammatory cells through the walls of blood vessels into the injury site; changes in biosynthetic, metabolic, and catabolic profiles of many organs; and activation of the immune system cells as well as of complex enzymatic systems of blood plasma. Of course, the degree to which these events occur is usually proportional to the severity and the extent of the injury, and inflammation ends when the inflammatory stimulus is eliminated and removed, otherwise the phase of chronic inflammation.

1.1.3. Inflammatory mediators

Inflammatory mediators can derive either from cells or from plasma proteins (Table 1). The cell-derived mediators are sequestrated inside of intracellular granules (e.g. histamine in mast cell granule), which are rapidly secreted by exocytosis or synthetized *de novo* in response to a stimulus. The main cells that produce or secrete this type of mediators are platelets, neutrophils, monocytes/ macrophages and mast cells; however, mesenchymal cells, including endothelium, smooth muscle and fibroblasts, are also able to do it if previously induced. Once activated and released from the cell, most of the inflammatory mediators have a short period of action, being quickly degraded or inactivated by enzymatic activity. Thus, there is a control and equilibrium system that regulates the inflammatory mediators activity (Kumar et al. 2009). On the other hand, plasma-derived mediators (e.g. complement proteins) are produced typically in the liver and are found as inactive precursors in circulation that must be activated by proteolysis, in order to acquire biological properties.

Table 1 – List of inflammatory mediators and their actions (adapted from Kumar et al. 2009).

Mediators	Sources	Action
	Cell-derived	
Cytokines		
TNF and Interleukins	Macrophages, endothelial cells, mast cells	Local endothelial activation (expression of adhesion molecules), fever/pain/anorexia/hypotension, decreased vascular resistance (shock)
Chemokines	Leukocytes, activated macrophages	Chemotaxis, leukocyte activation
Histamine	Mast cells, basophils, platelets	Vasodilation, increased vascular permeability, endothelial activation
Serotonin	Platelets	Vasodilation, increased vascular permeability
Prostaglandins	Mast cells, leukocytes	Vasodilation, pain, fever
Leukotrienes	Mast cells, leukocytes	Increased vascular permeability, chemotaxis, leukocyte adhesion and activation
Platelet-activating factor (PAF)	Leukocytes, mast cells	Vasodilation, increased vascular permeability, leukocyte adhesion, chemotaxis, degranulation, oxidative burst
Reactive oxygen species (ROS)	Leukocytes	Killing of microbes, tissue damage
Nitric oxide	Endothelium, macrophages	Vascular smooth muscle relaxation, microbes killing

Plasma protein-derived		
Complement products (C5a, C3a, C4a) **Kinins** **Proteases activated during coagulation**	Plasma (produced in liver)	Leukocyte chemotaxis and activation, vasodilation (mast cell stimulation); Increased vascular permeability, smooth muscle contraction, vasodilation, pain; Endothelial activation, leukocyte recruitment

The complement system is composed by more than 20 proteins and proteases that are activated in cascade (Forneris, Wu, and Gros 2012). Usually, this system has performance in both innate (natural resistances with which a person is born – e.g. epithelial barriers, dendritic cells) and adaptive immunity (acquired over time – naturally or artificially; and passive or active) with regard to defense against microbial pathogens. Their activation leads to the formation of complement proteins cleavage products, that stimulate the vascular permeability, chemotaxis and opsonization - process of coating a particle, such as a microbe, to target it for phagocytosis (Kumar et al. 2009). The main functions of these components are showed in Figure 3.

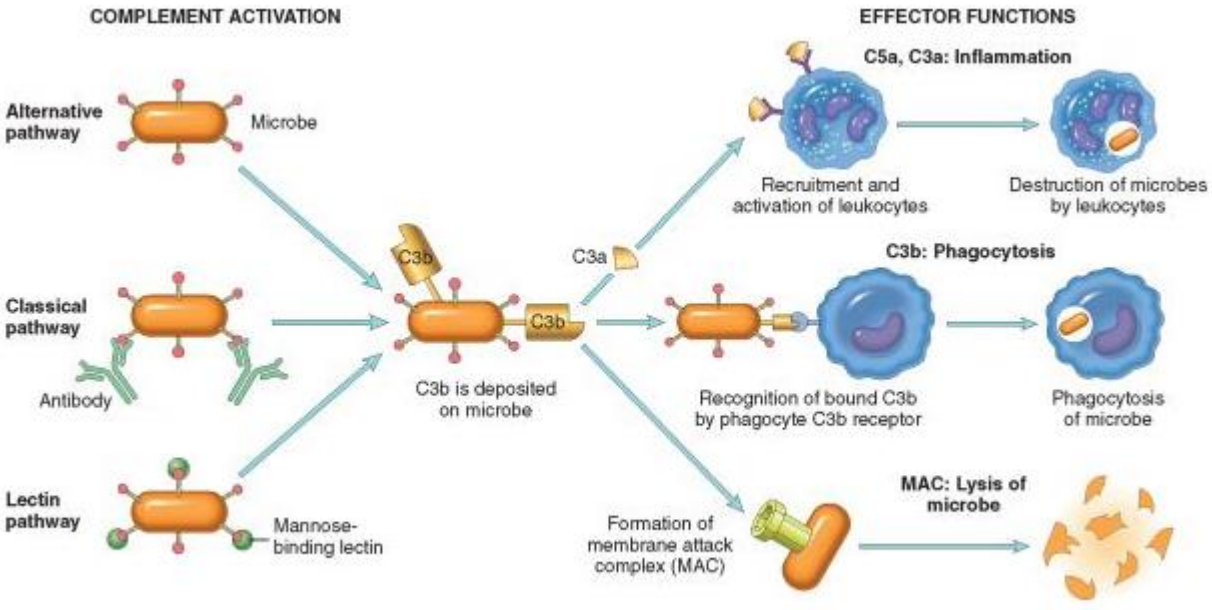


Figure 3 – The complement system. The complement system activation by classical, alternative or lectin pathways leading to breakdown products of C3 (the most abundant protein), which will trigger additional responses. All of these promote the formation of C3 convertase that convertes C3 into two functionally distinct fragments: C3a, which is released; and C3b, which is attached to the cell and, then, binds to the previously generated fragments to form C5 convertase, which in turn cleaves C5 to release C5a and leave C5b attached to the cell surface. C3a and C5a are the most important complement mediators of the inflammatory process. Functions of the several constituents involved are also indicated (taken from Kumar et al. 2009).

Due to their importance in the inflammatory process, cytokines will be now discussed in further detail.

1.1.3.1. Cytokines

Cytokines are a family of small peptides, comprising interleukins (ILs), interferons, growth factors, chemokine family and tumour necrosis factor family (TNF). These molecules are produced by numerous cell types, mainly by activated lymphocytes and macrophages at sites of inflammation (Kumar et al. 2009). In the central nervous system (CNS) specific brain cells (including microglia and astrocytes) are responsible for cytokines production upon activation (Tuppo & Arias 2005; Tambuyzer et al. 2009) (Section 1.4.1.). These cell-derived mediators regulate the intensity and the duration of the immune response (Heneka 2006) and their levels are in general increased in the inflammatory states (Tuppo and Arias 2005).

Until now, there is no unified cytokine classification system, with some authors including some interleukins in the chemokine family (e.g. IL-8) due to their functional properties. Moreover, some authors also categorize the cytokine class in two groups: pro-inflammatory cytokines that are associated with inflammation progress and, consequently, with tissue damage (which under this categorization includes IL-8); while anti-inflammatory cytokines are responsible to limit inflammation, by preventing injurious events on the organism (Heneka and O'Banion 2007).

In the present dissertation focus will be given to the interleukins and chemokines classes.

1.1.3.1.1. Interleukins

Currently, 37 interleukins have been identified and are numbered from 1 to 37, according to the order of their discovery. Thus, interleukins are a large group of immunomodulatory proteins that elicit a wide variety of responses in cells and tissues. These proteins bind to specific receptors located on cell surface, which act mainly on a paracrine or autocrine fashion. Depending on the ligands involved, particular signaling cascades can be activated, associated with growth modulation, differentiation and activation during an immune response. For instance, IL-1β is produced by several cells, including macrophages, monocytes, lymphocytes, microglia, neutrophils, fibroblasts, acting on T cells, fibroblasts, epithelial and endothelial cells, that in turn, induces pro-inflammatory proteins, hematopoiesis and differentiation of T-helper cells. IL-6 is produced by endothelial cells, fibroblasts, monocytes/macrophages. This protein acts on hepatocytes, leukocytes, T cells and B cells, and promote the synthesis of acute phase

proteins, leukocytes activation, T cell differentiation and activation, B cell differentiation and production of immunoglobulins (Akdis et al. 2011).

1.1.3.1.2. Chemokines

Chemokines are small peptides that act as chemoattractants, playing a crucial role in cellular migration and intercellular communication in normal tissues but also during inflammation (Kumar et al. 2009**;** Tambuyzer et al. 2009). Some chemokines are produced transiently in response to inflammatory stimuli, leading to the leukocytes recruitment to the damaged sites, whereas other chemokines are constitutively produced in tissues (Kumar et al. 2009).

The chemokine family consists of over 50 different molecules that confer chemotaxis, tissue extravasation, and modulation of leukocyte function during inflammation (Owens et al. 2005). These proteins can be divided into subfamilies on the basis of structural motifs. The CXC subclass of chemokines is considered one of the two major chemokine subfamilies and its members (e.g. IL-8) are primarily chemotactic for neutrophils and endothelial cells. The conserved glutamate–leucine– arginine (ELR) motif, within the receptor-binding domain of these proteins, distinguishes them from non-ELR CXC chemokines (such as IP-10), which primarily attract activated T cells (Strieter et al. 1995). The CC chemokine subfamily usually contain four cysteines (a small number can contain six cysteines) and two N-terminal adjacent cysteine. This group includes MIP-1α, MCP-1, and RANTES, do not affect neutrophils but are chemotactic for monocytes/macrophages, T-lymphocytes, basophils and eosinophils. IL-8 is produced by monocytes, macrophages, neutrophils, lymphocytes, endothelial cells, epithelial cells and fibroblasts. It exerts chemotactic functions for neutrophils, NK cells, T cells, basophils and eosinophils (Akdis et al. 2011). Although this protein is designed as an IL, it has been recently renamed due to its chemotactic function as CXCL8. However, this term is not commonly used in the literature.

Seven transmembrane, G-protein-coupled cell-surface receptors mediate the biological activities of chemokines and these receptors are named according to their chemokine subfamily classification. Until now or to date, five CXC receptors (CXCR1 to CXCR5) and nine CC receptors (CCR1 to CCR9) are known (Heneka and O'Banion 2007).

1.2. Neuroinflammation

Neuroinflammation is a local tissue response to injurious stimuli in the CNS and is characterized by glial reactivity, induction of cytokines release, and vascular permeability. In this process the typical inflammatory features (redness, edema and pain) previously described does not occur.

Regarding the brain defense against injuries or pathogens invasion, several inflammatory mechanisms are activated aiming the production of a variety of inflammatory mediators. These molecules are generated by brain cells previously activated, including microglia, astrocytes and neurons. The inflammatory process may pass to a longer term chronic phase (Section 1.6.) and impact on CNS functions. Indeed, chronic inflammation can be a key factor in the development of neurodegenerative diseases, among which AD, multiple sclerosis, Parkinson's disease, Huntington's disease (HD), amyotrophic lateral sclerosis (Frank-Cannon et al. 2009; Graeber, Li, and Rodriguez 2011).

1.2.1. Cellular and molecular mediators

Several molecular mediators are involved in neuroinflammation, mainly cytokines, which are produced by activated brain cells. As mentioned, these molecules play an important role in neurodegenerative diseases, promoting inflammatory processes in CNS. There are evidences that inflammatory cytokines and others molecules such as the complement system proteins, appear to play significant roles in the neuroinflammatory process (Reale et al. 2010). The mechanisms underlying to cytokines involvement will be further detailed, in particular to AD, in Section 1.3.

Microglia are the resident immune cells of the brain that support and protect neuronal functions. They are derived from monocyte precursor cells during embryogenesis, constitute around 10% of the cells in the nervous system, and represent the first line of defense against any brain tissue injury (Sastre et al. 2006; Tambuyzer et al. 2009**;** Rubio-Perez & Morillas-Ruiz 2012**;** Meraz-Ríos et al. 2013). Although microglia have neuroprotective and phagocytic functions, they can also have neurotoxic effects (Lee et al. 2010) when overstimulated. They are present in the CNS, where the white matter generally contains fewer microglia than the gray matter (Tambuyzer et al. 2009). In the absence of brain injury, microglial cells are in the inactive state and exhibit a small soma with branching processes presenting a resting ramified phenotype. Under pathological conditions (neurodegenerative disease, stroke and tumor invasion) they become activated undergoing several morphological changes, acquiring amoeboid form, decreased branching and increased soma growth, displaying a wide variety of specific cellular surface markers (Tambuyzer et al. 2009; Meraz-Ríos et al. 2013).

According to several authors, in addition to microglia, also peripheral macrophages are also able to perform phagocytosis and initiate innate immune response (Rezai-Zadeh et al. 2009; Gate et al. 2010). Their recruitment into the CNS is made by the release of specific cytokines during microglial and astrocytic activation, being able to cross the undisrupted blood-brain barrier (BBB).

Both microglia and macrophages, recognize foreign substances and pathogens through pattern recognition receptors (PRRs). Toll-like receptors (TLRs), nucleotide-oligomerization binding domain (NOD) proteins and C-type lectin receptors are included in the PRRs class. The interaction between these receptors and pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) promotes the beginning of the cellular defense mechanisms (Sterka & Marriott 2006; Rubartelli & Lotze 2007), which lead to production and release of pro-inflammatory cytokines (as IL-1β, IL-6, TNF-α, and INF-γ (interferon-γ)), chemokines (IL-8; RANTES-regulated on activation, normal T cell expressed and secreted; MCP-1-monocyte chemoattractant protein-1; MIP-1α and β - macrophage inflammatory protein-1), reactive oxygen species (ROS) and complement factors (C1q, C3, C4 and C9). These compounds contribute to neuronal dysfunction and cell death, promoting a vicious cycle (Lee et al. 2010; Meraz-Ríos et al. 2013). Additionally, microglia also express receptors for advanced glycosylation end products (RAGE), several types of scavenger receptors, among others (Okun, Mattson, and Arumugam 2010).

Astrocytes are the most abundant glial cells present in the CNS and have several functional capacities, being responsible for brain organization and maintenance (Sofroniew and Vinters 2010). In addition, they provide biochemical support to the endothelial cells of the BBB, supply nutrients to the nervous tissue, maintain the ion balance, restore the brain and spinal cord when they suffer injuries and provide trophic support to neurons (Lee et al. 2010). During brain inflammatory process astrocytes can also produce and secrete pro-inflammatory mediators and have been proposed to contribute to neuropathology underlying cognitive deficits.

Recent evidences demonstrate that neurons, by themselves, can generate inflammatory molecules, being a source of complement molecules, cyclooxygenases 2 (COX2)-derived prostanoids, inducible nitric oxide synthase (iNOS), C-reactive protein, amyloid P, pentraxins, macrophage colony-stimulating factor, and cytokines such as IL-1β, IL-6 and TNF-α. Additionally, neurons have been reported to secrete cluster of differentiation 22 (CD22), which inhibits the production of pro-inflammatory cytokines by microglia (Lee et al. 2010). The chemokines produced by neurons can work as messengers between neurons and glial cells, aiding in intracellular brain communication processes (Haass & Selkoe 2007; Biber et al. 2008).

1.3. Dementia: focus on Alzheimer's disease

Dementia, a syndrome usually associated with many causes, is characterized by a progressive loss of intellectual and cognitive functions that impairs the successful performance of daily living activities. It is most frequent in the developed world and is becoming even more so as a consequence of life span increase, thus contributing to an augmented risk of the elderly population suffering from dementia. Among the clinical symptoms, memory is the most common cognitive ability lost with dementia, affecting 10% of people aged over 70 years and 20-40% of individuals aged over 85 years. In addition to memory, other mental faculties are also affected, such as language, visuospatial ability, calculation, judgment and problem solving. Neuropsychiatric and behavioral alterations are also present in many cases of dementia, resulting in depression, agitation, insomnia, hallucinations and disinhibition (Bird and Miller 2010). Treatment is generally supportive or directed at relieving symptoms, and is usually far from perfect. Dementia is now an area of intense scientific study, which brings the perspective of more effective therapies and adequate treatments for the different dementia types in the future.

Recognizing dementia is easy if clinical symptoms are severe, which are normally associated with the late stages of disease. However, it is much harder to distinguish early dementia from the forgetfulness due to anxiety or from the mild cognitive impairment (MCI), that often accompanies ageing (usually affecting memory for names and recent events), and does not necessarily progress to more severe disability (Wilkinson and Lennox 2005). Also, alterations of multiple capacities usually distinguish dementia from other disorders, such as amnesia and aphasia, which affect a single functional domain (memory and language, respectively).

Most forms of dementia are progressive in nature, increasing in severity over time. The age of onset and the progression rate of symptoms differ among the major dementing disorders. Most have an insidious onset and develop slowly, sometimes over a period of many years, even before clinical manifestation of the symptoms. These include pathologies such as AD, HD and frontotemporal dementia (FTD).

In Europe, 7,3 million citizens suffer from dementing disorders and in Portugal over 153.000 people are affected. As life span is increasing, specialists predict that this value will duplicate in 2040 (http://www.alzheimerportugal.org). Presently, estimates indicate that there are nearly 36 million people with dementia worldwide (http://alzheimers.org.uk). Approximately 1% of the population is affected at age of 60-65 years, rising to 10–35% in those over 85 years old. Of the patients with late onset dementia (>65 years), about half have AD, 16% Vascular dementia and 30% other forms of dementia, such as dementia with Lewy bodies (DLB) and FTD (Lobo et al. 2000). In particular, for the Portuguese population, AD was also one of the most common forms of

dementia in a study realized in rural and urban areas from Northern of Portugal (Nunes et al. 2010).

AD was firstly described by the German psychiatrist and neuropathologist Alois Alzheimer in 1907 (Lee et al. 2010; Rubio-Perez & Morillas-Ruiz 2012). It is the most common form of dementia (50 to 75% of all cases) and age-dependent neurodegenerative disorder (Lee et al. 2010; Davinelli et al. 2011; Meraz-Ríos et al. 2013). It is often considered a multifactorial disease involving multiple molecular mechanisms (Davinelli et al. 2011).

Clinical signs suggestive of AD pathology include gradual memory loss, progressive cognitive impairment, decline of spatial and temporal orientation, loss of acquired skills, and emotional and behavioral disturbances (Glass et al. 2010; Lee et al. 2010). After a few years, approximately 5, all aspects of intellectual function are affected and the patient become frail and unsteady, generally requiring a full-time caregiver. Pneumonia is the principal cause of death in these patients (Castellani, Rolston, and Smith 2010). Recently, it has been suggested that the pathological process of AD initiates decades before the appearance of typical symptoms (Kim et al. 2011), and, generally, the clinical duration of the disease is around 8 to 10 years. It is estimated that AD affects around 27 million worldwide, 7-8 million in Europe, 90.000 individuals in Portugal (Rubio-Perez & Morillas-Ruiz 2012; http://www.alzheimerportugal.org). Approximately 8.4% of the AD patients have around 85 years or more, representing a growing public health problem as life expectancy increases (Davinelli et al. 2011).

1.3.1. AD molecular basis and histopathological alterations

Extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFT) are the two major neuropathological hallmarks of AD (Figure 4) (Tavee & Sweeney 2010**;** Davinelli et al. 2011). Brain autopsy of typical AD patients reveals these lesions and macroscopic cerebral atrophy (reduction of brain volume), as a cause of neuronal and synapse degeneration (Heneka 2006**;** Lee et al. 2010)**.** Usually, these lesions are present in specific brain regions implicated in learning and memory processes, such as temporal, parietal and frontal cortex as well as the hippocampus and amygdala. The affection of these areas, can explain in part the clinical symptoms observed in AD patients (Heneka 2006). Indeed, the presence and distribution of the NFT, SP and synaptic degeneration correlates with the degree of cognitive decline (Shankar and Walsh 2009). Other pathologic events such as, reactive gliosis, microglial activation, and neuroinflammation (Section 1.3) are also found in AD brains.

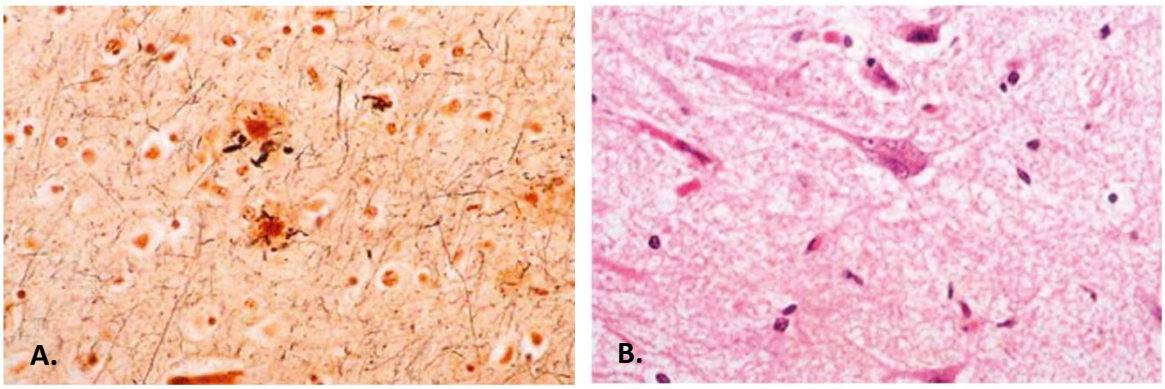


Figure 4 – Neuropathological hallmarks of AD. **A.** Senile Plaques (SP) mainly composed of aggregates of amyloid-β peptides (silver stained); **B.** Neurofibrillary Tangles (NFT) inside the neurons, resulting from Tau protein hyperphosphorylation (silver stained) (taken from Tavee & Sweeney 2010).

1.3.1.1. Senile plaques

SP (Figure 4 A.) are extracellular deposits mainly composed by aggregates of amyloid β (Aβ), which is a peptide that derives from the proteolitic cleavage of the Alzheimer´s Amyloid Precursor Protein (APP). APP can suffer proteolitic cleavage by the amyloidogenic or non-amyloidogenic pathways. In the amyloidogenic processing (Figure 5) Aβ is produced, by the sequential cleavage of APP by β-secretase (mainly beta-site APP cleaving enzyme 1 (BACE1) in neurons) and the γ-secretase complex (Heneka 2006; Benton 2011; Davinelli et al. 2011); while in the non-amyloidogenic pathway α-secretase precludes Aβ formation (O'Brien and Wong 2011).

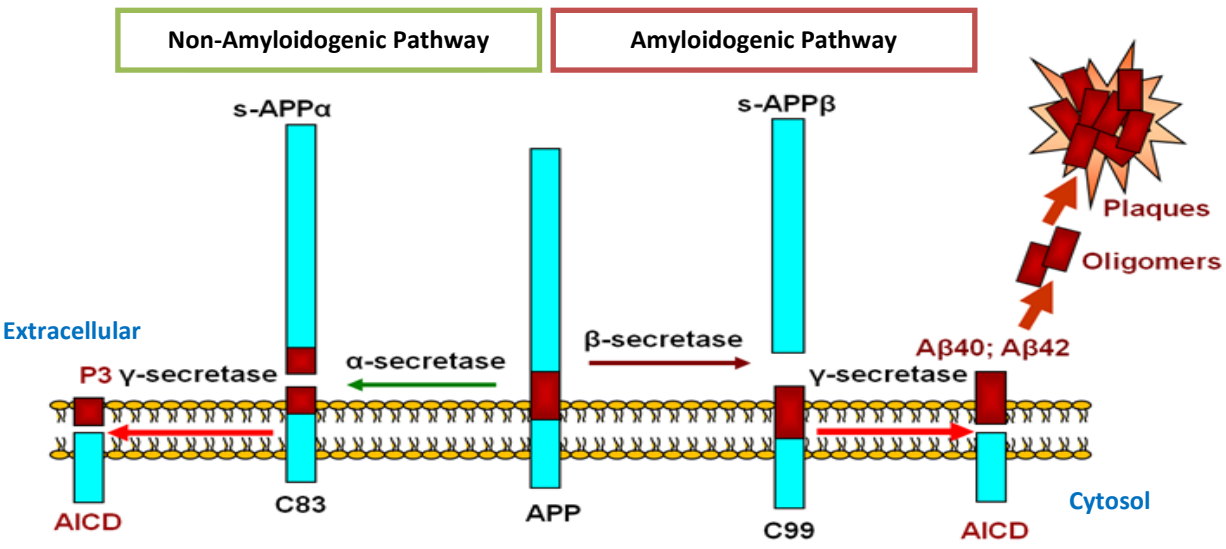


Figure 5 - APP processing and Aβ accumulation. APP can be cleaved by two different pathways. In the non-amyloidogenic pathway α-secretase originates sAPPα and C83, and the small peptide p3. In the amyloidogenic pathway β-secretase generates sAPPβ and C99. C99 is a substrate for γ-secretase, generating Aβ and, consequently, their accumulation in senile plaques (adapted from Zhang 2012).

$A\beta_{40}$ and $A\beta_{42}$ are the most common forms of Aβ, which are constituted by 40 or 42 amino acids, respectively (Heneka 2006). The $A\beta_{42}$ peptide is less soluble, has higher propensity to form aggregates and is more neurotoxic relatively to $A\beta_{40.}$ Of note, factors affecting normal APP processing, including abnormal phosphorylation, oxidative stress and Aβ itself, can contribute to abnormal Aβ production (Rebelo et al. 2007; Henriques et al. 2010).

Several studies support the amyloid hypothesis, which states that increased Aβ production and accumulation is the first event that triggers a pathogenic cascade that will lead to synaptic dysfunction, abnormal protein phosphorylation, apoptosis, oxidative stress and inflammation processes. As a consequence, neuronal function is affected, culminating in neurodegeneration typical of AD (Masters et al. 2006; Jakob-Roetne & Jacobsen 2009; Chow et al. 2010). In accordance with this theory, genetics, age and environmental factors can contribute to the imbalance between Aβ production and its clearance.

1.3.1.2. Neurofibrillary Tangles

NFT consist of intraneuronal aggregates of hyperphosphorylated forms of Tau protein (Figure 4 B.) (Heneka 2006; Glass et al. 2010). Tau is a microtubule-associated protein, that interacts with cytoskeleton proteins (such as actin) promoting microtubule assembly and stability, as well as regulating the intracellular vesicles and organelle traffic. In AD, the abnormal phosphorylation of Tau and their dissociation from microtubules leads to their breakdown into NFT and paired helical filaments (PHF) (Benton 2011), which in turn results in neuronal degeneration (Davinelli et al. 2011). With the disturbance of the tau-microtubule binding equilibrium, there is a resulting increase in the cytosolic unbound levels of tau as well, and consequently an increased likelihood of protein misfolding and subsequent aggregation as neuropil threads in dystrophic neuritis and as neurofibrillary tangles (Craig-Schapiro, Fagan, and Holtzman 2009). This fact could explain Tau increased levels found in CSF, since this protein is released from degenerating neurons and subsequent diffusion into this biological fluid.

In essence, alterations in the signaling cascades that lead to abnormal proteins phosphorylation or aggregation can potentially contribute to both SP and NFT formation. These alterations will interfere with normal neuronal function and integrity leading to degeneration typical of AD. Both histopathological alterations are related with the disease clinical manifestations progression as shown in Figure 6 (Citron 2004; Craig-Schapiro et al. 2009; Jakob-Roetne & Jacobsen 2009).

At preclinical AD stage there is already an abrupt increase of amyloid plaques formation, in contrast with a prompt decrease of neuronal integrity. However, at this phase AD pathology cannot be diagnosed since there are no clinical symptoms manifestations. It is only, in advanced phases when the severity of neurodegeneration already took place and the clinical symptoms manifestations are already evident, that AD can be detected.

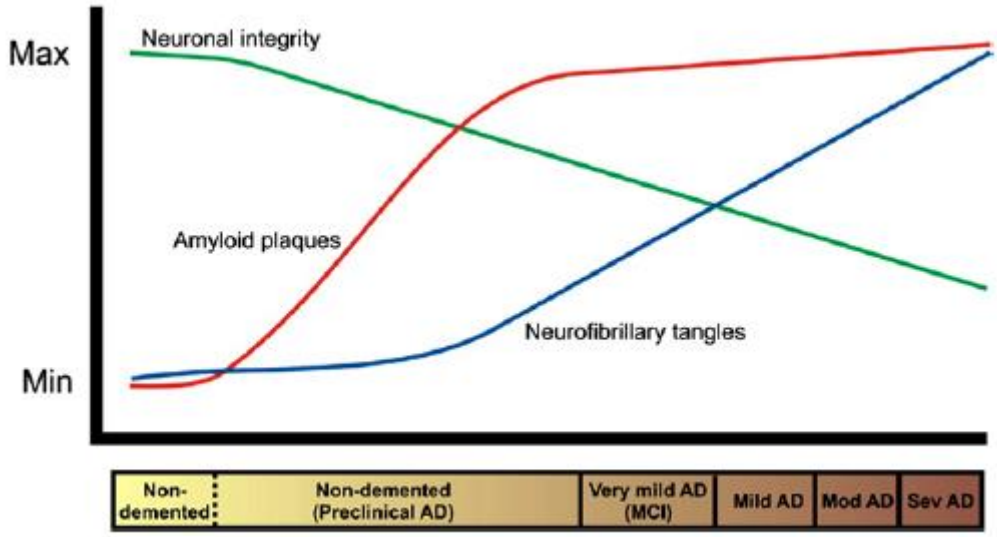


Figure 6 - Relationship between the neuropathology development and clinical changes of AD (taken from Craig-Schapiro et al. 2009).

1.3.2. Genetic basis and risk factors of Alzheimer's disease

Although the etiology of AD remains unclear, various risk factors have been associated with the disease, including genetic (mutation and polymorphisms), biologic and environmental factors. AD can be classified into two forms: early-onset AD (EOAD) and late-onset AD (LOAD).

In the EOAD, clinical symptoms start before 65 years. It represents less than 5% of all AD cases and is associated with hereditary genetic factors. APP, Presenilin 1 (PSEN1) and Presenilin 2 (PSEN 2) have been genetic factors involved in AD (Davinelli et al. 2011), which are located in different chromosomes while PSEN1 and PSEN2 are localized on chromosome 14 and 1, respectively. APP is localized in chromosome 21, explaining why individuals with trisomy 21 have a higher risk to develop AD (Thinakaran and Koo 2008). These mutations share a common biochemical pathogenic pathway, converging on

increased Aβ peptide production, in particular the $A\beta_{42}$ form (Citron et al. 1997; Davinelli et al. 2011; De Strooper, Iwatsubo, and Wolfe 2012).

On the other hand, LOAD is the most common form of AD affecting individuals over 65 years old. It has a sporadic origin and accounts for more than 95% of the total cases, which in time are triggered by normal aging neurodegeneration and diverse genetic and environmental risk factors. In this form, onset and progression of disease are insidious.

Other factors have also been associated with sporadic cases such as decreased brain capacity and reduced mental and physical activity during life (Gatz et al. 2006). As mentioned, sporadic cases of AD can also be associated with increased genetic susceptibility to develop the disease, being the most well documented risk factor the Apolipoprotein E gene (APOE). The ε2, ε3 and ε4 are the majors allelic variants of this protein and several studies have demonstrated that the APOE ε4 allele is the strongest genetic risk factor to AD development, since it is related, for instance, with increased propensity for Aβ aggregation (Herukka et al. 2007**;** Davinelli et al. 2011; Ryu et al. 2012).

The comprehension of the molecular mechanisms underlying the disease pathogenesis are useful not only to understand the genetic cases but also the sporadic form, since increased Aβ production and accumulation into SP is a common feature in both cases.

1.3.3. AD diagnosis: clinical, neurochemical and genetics

The clinical and pathological overlap among neurodegenerative disorders represents a challenge to diagnosis specificity (Reilly et al. 2010). In order to overcome misdiagnosis of dementia, cognitive evaluation tests (for exclusion of other dementias), neuroimaging exams, and genetic testing can be carried out. More recently, a neurochemical-based diagnosis consisting on the evaluation of a triplet of CSF biomarkers has been used in many European countries. The later can assist clinical evaluation and improve differential diagnosis of dementia, in particular AD from other forms of dementia (Craig-Schapiro et al. 2009; Lewczuk et al. 2009; Zetterberg et al. 2010).

The **clinical diagnosis** comprises cognitive and behavior assessment, in which the attention, concentration, language, memory and learning skills are evaluated. Mini mental (MM) is a commonly used questionnaire to evaluate the cognition and functional status, to check the existence of cognitive impairment and to monitor disease development (McKhann et al. 1984; O'Bryant et al. 2008; Eschweiler et al. 2010). It is an easily performed 30-point test that contains orientation, working memory (e.g., spell world backwards), episodic memory (orientation and recall), language comprehension, naming and copying tests (Eschweiler et al. 2010). However, this clinical diagnosis is not

100% conclusive requiring supplementary exams to improve diagnostic accuracy relatively to cognitive evaluation.

Several **neuroimaging tests** such as magnetic resonance imaging (MRI), single photon emission computerized tomography (SPECT) and positron emission tomography (PET) are applyied, providing relevant clinical results. The MRI presents higher resolution without exposing the patient to ionizing radiation, being useful to rule out other conditions that may cause symptoms similar to AD, detecting tumors, evidence of small or large strokes, damage from severe head trauma or a buildup of fluid in the brain (Frisoni et al. 2010). The techniques of SPECT and PET are able to study the cerebral perfusion and to measure brain energy metabolism. More recently, both PET and SPECT have been used to quantify Aβ in the brain taking advantage of the chemical Pittsburgh Compound-B (Klunk et al. 2004; Davinelli et al. 2011; Tartaglia et al. 2011). Nonetheless, many of these tests detect late features of AD, as brain atrophy of specific regions, when histopathological alterations are already present and cognitive impairment is already evident.

With regard to **neurochemical diagnosis** of AD, it has been identified and established a panel of biomarkers found in patients' CSF (Craig-Schapiro et al. 2009). CSF is in direct contact with the CNS, thus it becomes a favorable body fluid to be used in dementia diagnosis since it can reflect the biochemical and metabolic changes during the course of a neurological disease. $A\beta_{42}$, Phospho-Tau (P-Tau) and Total-Tau (T-tau) are the main altered biomarkers found in CSF of MCI or AD patients. The decreased $A\beta_{42}$ levels in CSF were related to the amyloid accumulation in the brain and the increase in P-Tau and T-Tau levels were related to neuronal damage or degeneration (Craig-Schapiro et al. 2009; Grimmer et al. 2009; Hampel et al. 2010; Paternicò et al. 2012). The combined use of $A\beta_{42}$, P-Tau and T-Tau CSF levels improves distinction between different forms of dementia and, in addition, are useful to predict the conversion from MCI to AD with elevated sensitivity and specificity (Craig-Schapiro et al. 2009; Davinelli et al. 2011). Patients with MCI have an elevated risk to develop AD (Risacher et al. 2009; Kester et al. 2011). Thus, evaluation of CSF biomarkers can be helpful for detection of patients with progressive disorder. However, as the later panel of neurochemical biomarkers requires CSF collection by lumbar puncture which is an invasive process (Davinelli et al. 2011; Kroksveen et al. 2011), many studies are also focusing on the identification and validation of more peripheral biomarkers. Additionally to CSF biomarkers, also atrophy of medial temporal structures, genetic risk factors, such as APOE ε4 and the presence of SP have been seen as strong predictive factors of the progression from MCI to AD. Therefore, all these factors together may allow an early AD diagnosis, preventing or delaying the dramatic consequences of this disease (Herukka et al. 2007; Craig-Schapiro et al. 2009).

Relatively to the **genetic diagnosis**, as previously mentioned, some mutations were identified in different genes that are directly related to AD. To evaluate the genetic risk to develop AD, genetic approaches may be of benefit in suspected familial forms of dementia, in particular when a highly penetrant gene mutation is inherited in an autossomal dominant pattern. In this situation, genetic testing may be an advantage since the identification of the specific mutations in affected family members will confirm the dementia diagnosis (Atkins and Panegyres 2011), and may help in the delay of AD symptoms. However, as these usually represent a minor part of the total cases of AD, genetic testing is not a routinely used diagnostic tool, and less so for ApoE4 which is only considered a genetic risk factor for AD.

Nowadays, there is no cure for AD, thus all efforts are directed for improving the sensitivity and specificity of the diagnostic tools, and in developing new tools that would allow early diagnosis, a stage where the existence drugs could be more effective in delying the disease progression. Presently, AD diagnosis is performed in basis of detailed clinical history, cognition and functional status assessment, neuroimaging, and in laboratory testing (Biasutti et al. 2012).

1.4. Neuroinflammation in Alzheimer's disease

The inflammatory process has a fundamental role in pathogenesis of AD, in which signs of chronic neuroinflammation and altered levels of some cytokines have been reported (Frank-Cannon et al. 2009; Kim et al. 2011; Leung et al. 2013). In this pathology, the presence of SP and NFT is able to trigger a series of cellular events which culminate in an inflammatory response mediated by activated microglia and reactive astrocytes (Figure 7), in an attempt to clear the injurious components (Rojo et al. 2008; Frank-Cannon et al. 2009; Meraz-Ríos et al. 2013). These activated inflammatory cells are found near neurons, surrounding extracellular SP, and are capable of up-regulating certain pro-inflammatory mediators such as cytokines, chemokines, complement molecules, among other molecules which could promote neuronal dysfunction and, consequently, neuronal death (Rubio-Perez and Morillas-Ruiz 2012). Additionally, it also has been described numerous interactions between cytokines and SP components that can create vicious cycles, contributing to pathology development (Rubio-Perez and Morillas-Ruiz 2012).

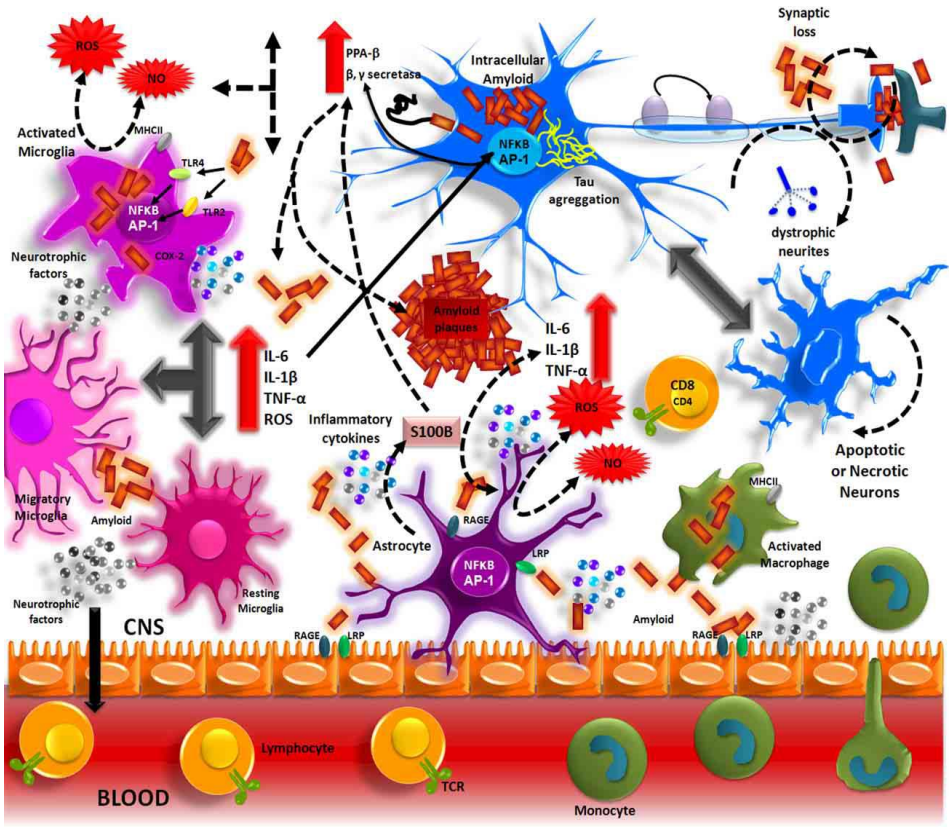


Figure 7 – Neuroinflammation process in AD. The Aβ aggregates promote microglia activation through TLRs and RAGE receptors. These receptors activate NF-kB and AP-1 transcription factors inducing the production of ROS and the expression of inflammatory cytokines such as IL-1, IL-6 and TNFα. These inflammatory compounds act on the neurons and stimulate the astrocytes, which amplify the pro-inflammatory responses, promoting neurotoxic effects. Adhesion molecules and chemokines are also produced, being responsible for the recruitment of peripheral immune cells. NFkB - nuclear factor-kappa B- dependent pathway; AP-1 – activator protein 1. (taken from Meraz-Ríos et al. 2013).

Initially, the inflammatory responses (mediated by pro-inflammatory mediators) are beneficial and necessary to prevent the neurotoxicity caused by the amyloid fragments. However, the anti-inflammatory components are also important in order to resolve the initial inflammatory response limiting the disease process. The persistent glial cells activation leads to chronic neuroinflammation that can contribute to disease progression and hastening of neuronal demise (Frank-Cannon et al. 2009).

1.4.1. Cellular and molecular inflammatory mediators in AD

Although, studies have proposed a role for the complement system in molecular mechanisms of neuroinflammation in AD (Shen & Meri 2003; Bohlson et al. 2007; Bénard et al. 2008; Maier et al. 2008; Ager et al. 2010), in this work emphasizes will be given to the role of inflammatory cytokines in this pathology.

Aβ induces microglia and astrocytes activation. Activated microglia (Figure 7) leads to increased expression of cell surface molecules of the major histocompatibility complex. Then, the adhesion of microglia to Aβ aggregates is mediated by scavenger receptors on cell surface, as TLRs and RAGE receptors (Lee et al. 2010; Meraz-Ríos et al. 2013). This binding induces the activation of extracellular signal-regulated kinases (ERK) and mitogen-activated protein kinases (MAPK) pathways, leading to pro-inflammatory genes expression and to the production of cytokines and chemokines (Sastre et al. 2006; Heneka & O'Banion 2007; Rubio-Perez & Morillas-Ruiz 2012). Among those are IL-1 and IL-6 but also IL-8, MCP-1 and RANTES.

Microglia can play a beneficial role against AD, since its activation reduces Aβ accumulation by increasing its phagocytosis, clearance and degradation. Additionally, they secrete soluble factors, such as glial-derived neutrophic factor (GDNF), which has been demonstrated to be beneficial for neurons survival (Heneka & O'Banion 2007; Rubio-Perez & Morillas-Ruiz 2012).

Astrocytes also provide a protective barrier between Aβ deposits and neurons, playing an important role in Aβ clearance and degradation (Sastre et al. 2006; Rubio-Perez & Morillas-Ruiz 2012). Similar to microglia, astrocytes are activated by Aβ aggregates through TLRs and RAGE-dependent pathways and also produce and secrete a variety of pro-inflammatory molecules. The presence of $A\beta_{42}$ inside of astrocytes is a consequence of the phagocytosis of local degenerated dendrites and synapses (Sastre et al. 2006; Meraz-Ríos et al. 2013). However, under certain conditions related to chronic stress, the intense activation of microglia and astrocytes may not be beneficial, prolonging neuroinflammation and contributing to neurotoxicity mediated by expression of inflammatory mediators, such as iNOS, ROS, NO (Heneka and O'Banion 2007; Meraz-Ríos et al. 2013). Recently, it has been proposed that astrocytes could also be a source of Aβ, because they overexpress the APP β-secretase in response to chronic stress (Rossner

et al. 2005). Hence, under stress conditions, astrocytes can potentially exacerbate neuronal damage and accelerate disease progression by intensifying neuronal death due to increasing Aβ production.

Aβ leads to increased levels of cytokines, including IL-1β, IL-6, TNF-α, MIP-1α and MCP-1 upon microglia activation (Meda et al. 1999; Sastre et al. 2006). In addition, Aβ was also able to stimulate NFkB, which is necessary for cytokine production. Pro-inflammatory factors produced by neurons can also contribute to neuronal damage and, consequently, to AD pathogenesis by triggering neuroinflammatory processes; nevertheless, neuronal generation of TNF-α and low concentrations of NO, may be considered a defense mechanism against local inflammatory reactions, since they confer neuroprotection (Heneka and O'Banion 2007). Therefore, the function of these brain neuroimmune modulators oscillates between neuroprotective and neurodegeneration effects, depending on the AD stage.

In a chronic stage, the continued release of many of these molecules mediators, may also impact on AD by affecting APP expression, and processing, Aβ deposition, Tau phosphorylation, NFT formation and neurodegeneration.

For instance, *in vitro* studies showed that IL-1β is able to activate the MAPK-p38 pathway which can phosphorylates Tau protein (Li et al. 2003; Rojo et al. 2008). Further, both interleukins were shown to affect up-regulate activity of the Ciclin-dependent kinase (CdK)5/p35 complex, the main protein kinase involved in Tau phosphorylation process (Rojo et al. 2008). More recently, Kitazawa and their colleagues (2011) reported that the inhibition of IL-1β signaling in AD animal models decreased the activity of cdk5/p25 but also of glycogen synthase kinase (GSK)-3β, and p38-MAPK, leading to decreased P-Tau levels. Moreover, it was shown that IL-1β could be involved in increasing APP non-amyloidogenic cleavage, therefore decreasing Aβ production, due to α-secretase up-regulation (Tachida et al. 2008). Further, recent studies have shown that this cytokine can induce Aβ removal, suggesting a neuroprotective role for IL-1β in AD neuropathogenesis (Matousek et al. 2012).

IL-6 was overexpressed in brains of APP transgenic models, causing significant gliosis and decreasing Aβ deposition *in vivo* this was corroborated by the up-regulation of the glial phagocytic marker and stimulation of microglial phagocytosis of Aβ. Additionally, IL-6-induced neuroinflammation did not affect the APP processing in the transgenic models tested, suggesting a beneficial role of reactive gliosis by promoting Aβ clearance in early stages of AD (Chakrabarty et al. 2010).

IL-8 is an important chemokine for the recruitment of activated microglia to damage brain sites. IL-8 receptors are located in dystrophic neurites indicating that this chemokine is able to establish glial interactions with neurons and, thereby, contribute to neuronal damage (Kim et al. 2011) by excreting its pro-inflammatory actions. Alternatively, IL-8 can have a protective role against Aβ-induced neurotoxicity, by

stimulating the increase of brain-derived neurotrophic factors levels in human neurons (Ashutosh et al. 2011)

Further, oxidative stress induced by Aβ, can up-regulate RANTES expression in rat brain endothelial cells. Since, neurons treated with RANTES demonstrated increased cell survival by the increased in RANTES levels, may suggest a beneficial effect in AD neuroinflammation. However, how RANTES exerts its neuroprotective effects remain unclear (Tripathy et al. 2010).

1.4.2. Cytokine levels in AD

The levels of cytokines and other proteins associated with inflammatory processes have been extensively investigated of AD patients' blood and CSF to uncover mechanisms of neuroinflammation either in dementia or in the context of biomarker research. However, the studies that addressed the levels of the above mentioned molecular mediators in patient tissues and peripheral body fluids are controversial (For review see Brosseron et al. 2014). We will give emphasis to the interleukins and chemokines adreessed in our study.

- **IL-1β** – IL-1β levels were reported to be elevated in CSF of AD patients in comparison to controls, and unchanged in plasma samples of the same individuals (Blum-Degen et al. 1995). In contrast to this study, the plasma levels of IL-1β were found increased in AD patients (Licastro et al. 2000). In agreement, more recently IL-1β levels were shown to be increased in serum samples of MCI and AD patients comparatively to control subjects (Forlenza et al. 2009). Although there are still inconsistent data regarding to IL-1β levels, no study revealed down-regulation of this interleukin in AD pathology (for Review see Brosseron et al. 2014).

- **IL-6** – The levels of IL-6 were also measured in CSF and plasma samples. Similar to IL-1β, IL-6 levels in CSF were reported to be increased in AD patients while plasma levels were not significantly affected when compared to healthy individuals (Blum-Degen et al. 1995). Controversially, according to Kálmán et al. (1997), no changes could be detected for CSF of MCI and AD patients. Severe AD patients showed higher IL-6 levels comparatively to less severe AD and controls subjects. Moreover, serum IL-6 levels correlate with the severity of dementia in Down syndrome and in AD (Kálmán et al. 1997). IL-6 plasma levels were also found increased in patients with AD comparatively to controls (Licastro et al. 2000).

For both interleukins, the literature data is not consensual and additional studies should be carried out. Nonetheless, since several findings indicate that the plasma levels of IL-1β as well as IL-6 are altered in AD, the detection of these molecules, along with others, and the correct definition of their pattern might be useful for the monitoring of brain inflammation associated with AD (Blum-Degen et al. 1995; Licastro et al. 2000; Forlenza et al. 2009).

Chemokines are physiologically generated at basal levels in the healthy CNS, and recent studies have focused in their role, expression and receptors in neurodegenerative diseases, including in the AD associated neuroinflammation. As mentioned, chemokines are typically produced by microglia and astrocytes, while their receptors are present in neurons. This fact promotes the communication between glial cells and neurons establishing a local inflammatory response that could favor the Aβ phagocytosis in AD early stages (reviewed in Zilka et al. 2012). Nevertheless, in chronic inflammation, as is the case for AD, increased chemotaxis of the phagocytic cells is observed resulting in increased microglial recruitment around Aβ deposition, which can prompt neuronal demise (Sastre et al. 2006). Aβ stimulate chemokine release not only in microglia but also in astrocytes, neurons, and monocytes (Meda et al. 1999; Streit et al. 2001; Sastre et al. 2006; Ashutosh et al. 2011).

In particular, up-regulation of several chemokines, such as IL-8, MCP-1, RANTES, and MIP-1 (both α and β types), as well as chemokine receptors in CSF of AD patients were shown. However, in plasma samples, the reports showed no consensual results (Table 2).

- **IL-8** – It has been found increased in the CNS, in particular in the CSF and brain tissue of AD patients relative to controls. Contrary to the increases observed for this chemokine in the CSF of both MCI and AD patients, circulating plasma IL-8 levels decreased for both patients (MCI and AD) in comparison to control individuals (Kim et al. 2011). Nonetheless, some studies also report that plasma IL-8 levels do not change in AD (Leung et al. 2013).

- **MCP-1** (also named CCL2) – It was reported that plasma MCP-1 levels were unchanged when comparing controls and AD patients while increases of this chemokine were detected in CSF of AD patients. The increases in CSF levels correlated with cognitive decline (Westin et al. 2012). Alternatively, decreased plasma levels of this chemokine in AD patients relatively to healthy individuals were recently reported (Reale et al. 2012). Moreover, differences arise for MCI and early versus severe AD cases. MCP-1 levels were found significantly increased in plasma of MCI and mild AD patients but not in severe AD cases in comparison to controls (Kim et al. 2011). Consistently, elevated MCP-1 levels were also detected in serum samples of both MCI and early AD patients while lower levels were

reported for severe AD cases (Galimberti et al. 2006). These findings suggested that MCP-1 plasma/serum levels could be a useful biomarker to monitor the inflammatory process in AD.

- **RANTES (also named CCL5)** –RANTES showed elevated expression in the cerebral microcirculation of AD patients (Tripathy et al. 2010) and increased levels in peripheral biological fluids in AD (Reale et al. 2012). However decreases in mRNA expression of this chemokine in AD blood samples were also reported (Kester et al. 2012).

Table 2 summarizes the changes of these cytokines in different peripheral fluids and AD brains (for review see Brosseron et al. 2014).

Table 2 – Detection of cytokines levels in plasma, CSF and AD brains.

	Plasma	CSF	AD brains	References
Interleukins				
IL-1β	= ↑	↑	↑	(Blum-Degen et al. 1995; Licastro et al. 2000; Grammas and Ovase 2001; Forlenza et al. 2009; Leung et al. 2013)
IL-6	= ↑	↓↑	↑	(Bauer et al. 1991; Blum-Degen et al. 1995; Kálmán et al. 1997; Licastro et al. 2000; Grammas and Ovase 2001)
Chemokines				
IL-8	= ↓	↑	↑	(Ashutosh et al. 2011; Kim et al. 2011; Leung et al. 2013)
MCP-1	= ↓	↑	↑	(Grammas and Ovase 2001; Galimberti et al. 2006; Kim et al. 2011; Westin et al. 2012; Leung et al. 2013)
RANTES	↑	-	↑	(Tripathy et al. 2010; Kester et al. 2012; Reale et al. 2012)

In essence, cytokines are important to trigger immune response in CNS, being responsible for recruitment of microglia and astrocytes to the site of Aβ deposition releasing molecular inflammatory mediators and defining the extension of local inflammation. However, in general it has been proposed that their chronic production in advanced AD could be harmful and contributes to neuronal death. Controversial results still exist regarding the function, the effect and the levels of the mentioned cytokines in

AD neuroinflammation. These discrepancies could be related with inter-individual variances, collecting and processing of biological samples, lack of patients collective characterization and differences between the technical approaches of the studies. Hence, additional studies are needed to improve our knowledge in this field and to discover reliable inflammatory biomarkers for AD.

II. Aims of the Thesis

AD is a complex neurodegenerative disorder neuropathologically characterized by the presence of SP and NFT, synaptic loss and consequently neurodegeneration. The Aβ peptide is the major constituent of SP and plays a crucial role in AD pathology. Increased Aβ production and aggregation was associated with a series of pathogenic processes, which include, among others, activation of inflammatory responses that will contribute to neurodegeneration and potentially to gradual cognitive decline. Microglia are activated by Aβ aggregates leading to the expression of inflammatory cytokines. The initial inflammatory response is beneficial and necessary to prevent the neurotoxicity caused by amyloid fragments and to limit the disease progress. Generated cytokines act directly on the neurons and stimulate the astrocytes, and chemokines can also recruit peripheral immune cells to injured regions of the brain. Both mechanisms amplify the pro-inflammatory responses, which cause neurotoxic effects and contribute to neuronal death. In an advanced AD phase, due to the persistent activation of microglia, chronic neuroinflammation occurs. Chronic inflammation found in AD brains has been recognized as sign and a fundamental mechanism involved in pathological disease progression. Thus far, different inflammatory markers have been addressed in CSF and serum of AD patients, nonetheless conflicting results have been described. Since the identification of AD biomarkers will aid in the differential diagnosis of this disease from other dementia subtypes, the main aim of this project was to identify inflammatory biomarker candidates for AD and/or dementia. In particular, as CSF collection is an invasive procedure, investigation has been directed towards the search of noninvasive peripheral biomarkers. Therefore, the following specific aims were to:

- Evaluate the profile of inflammatory proteins in plasma samples of patients with cognitive decline, including a group of possible AD patients;

- Address the relation between the inflammatory biomarkers evaluated and the cognitive tests applied in our study population;

- Establish correlations between the inflammatory biomarkers analysed;

- Setup the experimental conditions to determine the effects of the most promising inflammatory candidates on APP processing.

III. Methods

3.1. Study subjects

For this study, plasma samples from control individuals and patients with cognitive decline were analysed. Patients were subgrouped based on the individual cognitive evaluation as described below (Section 3.1.2).

3.1.1. Sample collection

Plasma samples were collected and processed within 1h after collection, according to standard procedures in an EDTA tube (K2 EDTA with gel, 5 mL), to prevent coagulation. Once arrived at the laboratory, samples were centrifuged at 1800 g, for 15 minutes at 4^{o}C. Supernatant was transferred for a new tube and ressuspended. Samples were aliquoted (each with 500 µL) and frozen at -80°C.

3.1.2. Cognitive evaluation

Plasma samples were obtained from the group of 46 individuals, selected from the CBC Cohort project based on the cognitive evaluation criteria. Cognitive evaluation of individuals was carried out at several Centers for Primary Health Care in the Aveiro region. The project was approved by the ethics committee of the Regional Health Center - Coimbra, protocol number 012 804 of April 4, 2012.

The inclusion criteria for this study group were: age between 50-90 years, resident in the Aveiro region, with complaints that include objective memory impairment or other cognitive complains. The exclusion criteria were individuals undergoing chemotherapy or radiotherapy, psychiatric illness such as bipolar disorder, schizophrenia, and the use illicit drugs.

The cognitive tests applied to the study group were the Clinical Dementia Rating scale (CDR) (Hughes et al. 1982), the Mini-Mental State Examination (MMSE) (Folstein et al. 1975) and the Geriatric Depression Scale (GDS) (Mitchell et al. 2010).

According to the CDR scale: **0** indicates normal function; **0.5** indicates a transition level (termed very mild dementia); **1.0** indicates significant loss (almost always a clear correlation with dementia); **2.0** indicates loss of moderate cognitive function; **3.0** indicates severe loss. For this study cognitive dysfunction was considered when CDR ≥ 0.5.

The MMSE test allows patient stratification according to the education level: cutoff of **22** for 0-2 years schoolarship; **24** for 3-6 years; and **27** for more than 7 years (Morgada et al. 2009). Additionally, clinical routine questions were included to address other possible neurological pathologies.

Depressed individuals were excluded from the group using the GDS scale. The GDS test (Mitchell et al. 2010) consists of 15 questions, to survey for symptoms suggestive of depression, in which individuals with 0-5 positive questions were considered normal.

According to the cognitive evaluation, individuals were subdivided in 3 groups: a control group (negative for CDR and MMSE tests); a group with cognitive alteration but MMSE negative (CDR positive and MMSE negative); and a possible AD group (positive for both CDR and MMSE scales), in which 5 of 12 patients are clinically diagnosed with AD. Table 3 presents the age variable and total number (n) of the study group individuals.

Table 3 – Characteristics of age variables and n total from the study groups.

Plasma Samples			
Subgroups/ Characteristics	**CDR- MM-**	**CDR+ MM-**	**CDR+ MM+**
Age (years)			
Mean	76	74	79
Min-Max	65-82	70-84	72-88
Gender			
Male n	8	3	1
Female n	17	6	11
N total	25	9	12

3.2. Flow cytometry analysis

Flow cytometry is a fast, objective and quantitative method, useful for detection of cell surface markers, intracellular factors, cell-secreted factors, DNA content, among others. This process allows the measurement and counting of the physical and chemical characteristics of biological particles.

In this study, inflammatory proteins in peripheral biological samples were analyzed by flow cytometry, using a cytometric bead array (CBA). CBA allows the specific detection of soluble proteins (in this case cytokines and chemokines) in complex biological fluids. CBA is a multiplexed bead-based immunoassay, in which beads are couple with high-affinity antibodies for the markers of interest, allowing to quantify multiple proteins simultaneously in the same sample.

After bead incubation with the biological sample, phycoerythrin (PE)-conjugated antibodies were added, providing a fluorescent signal proportional to the amount of the bound analyte (Figure 8). Each bead population is classified with an alphanumeric

position indicating its position relatively to other beads in the CBA array, which allows the distinction of the fluorescent signal of each protein analyzed. It permits beads with different positions to be combined and used at the same time, creating a multiplex assay.

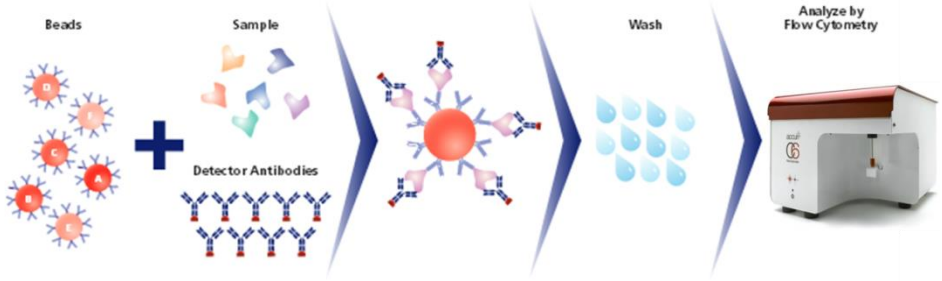


Figure 8 – Principle of the CBA Soluble Protein Flex Set System.

The IL-1β, IL-6, IL-8, MCP-1 and RANTES were the inflammatory proteins analysed in this work according to the manufactures' instructions (CBA Human Soluble Protein Flex Set System, from BD Biosciences). Depending on the number of tests in the experiment, a specific volume of each capture bead (1 µL/test) was mixed in 0,5 mL of Washer Buffer. The resulting solution was then centrifuged at 200 g for 5 minutes. Supernatant was removed and the mixed beads ressuspended in Capture Bead Diluent to a final concentration of 50 µL/test. Mixed beads solution was then added to samples. PE Detection Reagent was also dependent on the number of tests (1 µL/test) as well as the total volume of diluted PE Detection Reagent needed for the experiment, being that each test tube requires 50 µL of this diluted reagent. The PE Detection Reagent of each protein were mixed and diluted, before adding to samples, according to manufacturer's instructions. For quantitative analysis of these proteins, a standard curve was prepared as mentioned in Table 4.

Samples were analysed and acquired on Accuri C6 Flow Cytometer (BD Biosciences), using FCAP Array™ software to generate results in graphical and tabular format.

Table 4 – CBA standards preparation. List of standard dilution and respective concentration (pg/mL).

Standard dilution	Concentration (pg/mL)
No standard dilution (Assay Diluent only)	0
1:256	10
1:128	20
1:62	40
1:32	80
1:16	156
1:8	312,5
1:4	625
1:2	1250
Top Standard	2500

3.2.1. Data analysis

Statistical analyses were performed using the SPSS 21.0 software. The flowchart below represents the sequential steps for statistical analysis of the flow cytometry data. The Shapiro-Wilk test was used to examine normal distribution. Data analyses were carried out using non-parametric tests, since the normality test was not validated for our population. Comparisons between groups were performed applying the Kruskal-Wallis test. The graphs were presented in box plots format, enabling the observation of outliers in different groups of the study population, which were plotted as individual points. Additional statistical analysis was carried out using the Wilcoxon Signed Rank test for the 21 paired age- and sex-matched pairs. Furthermore, Spearman's Rank Order was used for correlation analysis between the inflammatory proteins, as well as to verify the correlation of the inflammatory proteins with CDR scores. To test if the results are or not significant, the null hypothesis (H_0) was imposed. With a confidence interval (CI) of 95%, $p<0.05$ was considered statistically significant, rejecting H_0.

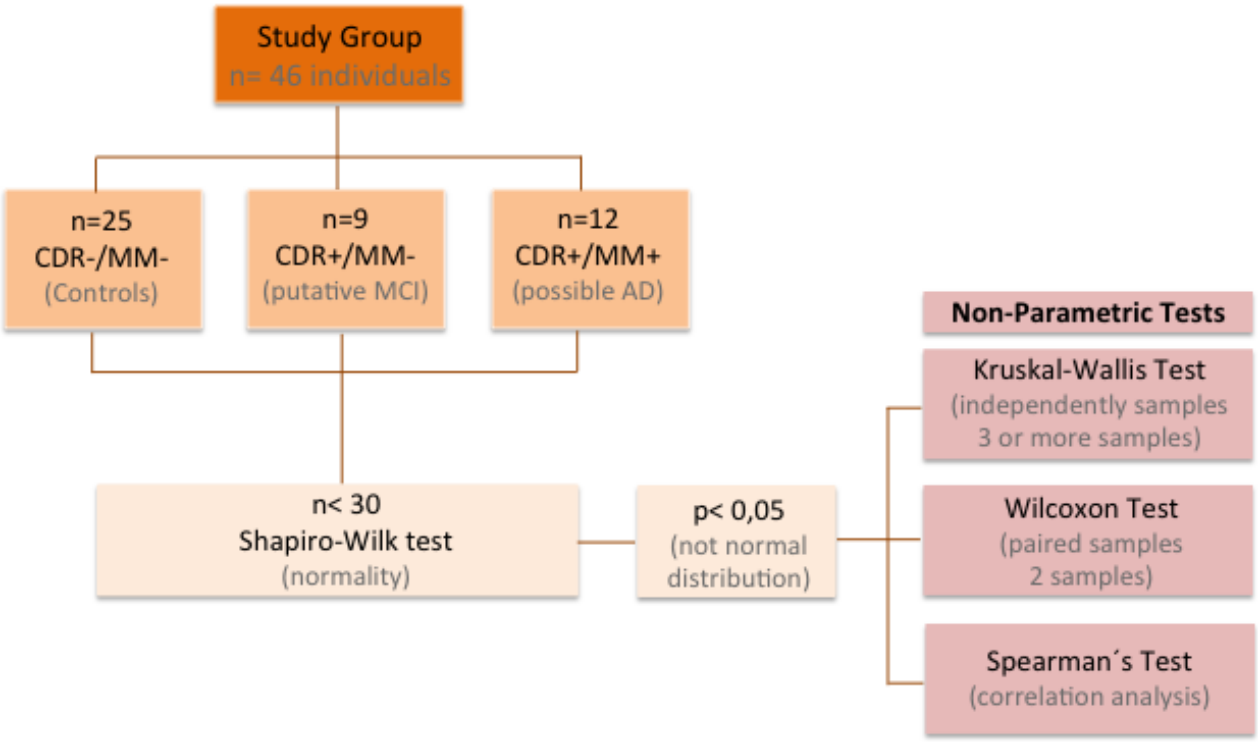


Figure 9 – Statistical analysis workflow for the study group.

3.3. Cell culture experiments

A set of experiments were carried out in SH-SY5Y cells. These are human cells derived from the original cell line SK-N-SH, isolated from a bone marrow biopsy of a neuroblastoma patient. SH-SY5Y cells were maintained in Minimal Essential Medium (MEM):F12 (1:1) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1% antibiotic/antimycotic mix. Cells were maintained in an atmosphere of 5% CO_2 at 37°C and split when 80-90% confluent. Cells were plated at a density of $5x10^5$ cells per well (12-well plates) or $1,3x10^5$ cells/cm^2.

3.3.1. IL-8 experimental procedures

Cells were incubated with crescent concentrations of IL-8 (BD Biosciences) for different periods of time, 6 and 24 hours. Lyophilized IL-8 was reconstituted in distilled water (20 µg stock), aliquoted and stored. For experimental purposes IL-8 stock solution was subsequently diluted in serum free culture medium at the final concentrations of 10, 50 and 100 µM and then added to cells.

3.3.2. Sample collection and immunodetection

After the appropriate treatments, cells lysates were collected in RIPA buffer. Samples were stored at -20°C. Protein determination content was performed using BCA assay (see below) and normalized protein samples were electrophoretically separated by 7.5% SDS-PAGE gels (Section 3.3.2.2.). Separated proteins were transferred onto a nitrocellulose membrane (Section 3.3.2.3.1.) followed by immunoblotting for the specific protein (Section 3.3.2.1.). Detection was carried out using a chemiluminescent method (Section 3.3.2.3.2.) and the resulting bands were quantified by densitometry (Section 3.3.3.).

3.3.2.1. Protein concentration determination

The bicinchonic acid (BCA) protein assay (Pierce) was used for the colorimetric detection and quantification of total protein concentration. This test is based on the capability of proteins to reduce Cu^{2+} to Cu^{+} in an alkaline environment (the biuret reaction). BCA produces a purple color in the presence of the reduced Cu^{+} ion that results from chelation of two molecules of BCA with one cuprous ion. These soluble complexes exhibit a strong absorbance that can be read at 562 nm.

The quantitative analyses were carried out using 5 µL of the collected cell lysates. To determine the total protein content in each sample a standard curve was prepared as

described in Table 5. Samples and standards were incubated with 200 µL of working reagent, which is prepared with 50 parts of Reagent A to 1 part of Reagent B. All samples were incubated at 37°C during 30 minutes, cooled to room temperature and immediately measured at 562 nm.

Table 5 – Standards used in BCA protein assay method. BSA, Bovine Serum Albumin solution (2mg/mL).

Standard	BSA (µL)	SDS 1% (µL)	Final Protein Mass (µg)
P0	-	25	0
P1	1	24	2
P2	2	23	4
P3	5	20	10
P4	10	15	20
P5	20	5	40

3.3.2.2. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method used to separate components of a protein mixture based on their molecular weight and negative charge. The principle of SDS-PAGE relies on the capacity for proteins to migrate through gel pores when submitted to an electrical field. The gel percentage and size depend on the molecular weight of the proteins to be separated. As proteins have different electrical charges that affect their mobility, SDS is usually added to protein samples and buffers to confer a negative charge to all proteins, ensuring protein migrate toward the positively charged anode. SDS is also used in combination with a reducing agent (mercaptoethanol) and heated to dissociate proteins before they are loaded on the gel. SDS also breaks up aggregates and non-covalently bound multimers. Gels comprise 2 phases, the non-restrictive large pore called stacking gel and the resolving gel with an acrylamide concentration of 7,5%. The gel was prepared and allowed to polymerize at room temperature for 45 minutes. Subsequently, the stacking gel solution was prepared and loaded on the top of the resolving gel, and left to polymerize at room temperature for 30 minutes.

The samples to be run on the gel were boiled in SDS gel loading buffer for 5 minutes to ensure protein denaturation. Precision plus protein standards Dual Color (BioRad) was used as marker. Proteins were separated electrophoretically at 90 mA for approximately 3 hours in a Hoefer electrophoresis system.

3.3.2.3. Western blotting analysis

Western blotting is the technique used for detection of specific proteins in complex samples like cell lysates, cell culture supernatants or body fluids. In this technique, proteins separated by SDS-PAGE were transferred to a solid membrane by the application of an electrophoretic field. This is a fast and efficient procedure and preserves the high-resolution separation of proteins by SDS-PAGE. In the membrane, proteins are suitable to detection by total protein staining or labeling of the proteins of interest with specific antibodies.

3.3.2.3.1. Transfer of Proteins from the membrane to a Solid Support

Proteins were electrophoretic transferred to a nitrocellulose membrane. The gel was placed in contact with a nitrocellulose filter and then sandwiched between Whatman 3 MM paper, two porous pad and two plastic supports. The nitrocellulose filter was placed toward the anode. An electric current of 200 mA was applied for at least 16 hours. After proteins transfer, the membrane was removed from the sandwich and allowed to dry at room temperature.

3.3.2.3.2. Immunological Detection of the Immobilized Proteins – Chemiluminescent Protein Detection

For APP detection the Rabbit polyclonal antibody anti-APP (APP C-Terminal) was used to detect full-length APP. The mouse monoclonal anti-APP (22C11, N-Terminal) was used to detect the APP N-terminal fragments (sAPP) in the conditioned medium.

Membranes were initially soaped in 1x TBS for 5 minutes. Blocking of possible non-specific binding-sites of the primary antibody was performed using 5% (w/v) BSA in 1x TBS-T solution. Subsequently, membranes were incubated with an unlabeled primary antibody direct against the target protein for 4 hours with agitation at room temperature plus overnight incubation at 4^{o}C. After washing with 1x TBS-T (3 times, 10 min each) membranes were incubated with the secondary antibody (coupled with horseradish peroxidase) for 2 hours. Membranes were additionally washed 3 times with 1x TBS-T before protein detection.

The detection method (Table 6) used was enhanced chemiluminescence (ECL) reagent. This method is based on the oxidation of the cyclic diacylhydrazide luminal that results in light emission. The membranes were incubated with the working mixture of the chemiluminescent detection reagent, for 1 minute at room temperature. The membranes were exposed to autoradiography films (Kodak) in an X-ray film cassette. Films were

exposed for different periods in order to optimize signal, developed and fixed with appropriate solutions (Kodak).

Table 6 – Antibodies and detection method used for protein immunodetection.

Protein	First Antibody	Species Reactivity	Secondary Antibody	Detection Method
APP C-Terminal	Rabbit anti-APP Dilution 1:1000 (Invitrogen)	Human, mouse, pig, rat	Peroxidase labeled anti-rabbit Dilution 1:5000 (Amersham Pharmacia)	ECL
22C11	Mouse anti-APP (N-Terminal) Dilution 1:250 (Boehringer)	Rat, human, monkey	Peroxidase labeled anti-mouse Dilution 1:5000 (Amersham Pharmacia)	Luminata™ Crescendo

3.3.2.4. Ponceau red staining of protein bands

Ponceau Red staining was applied as a loading control. This type of staining has been described as a fast, inexpensive, and nontoxic method and its binding is fully reversible in a few minutes (Romero-Calvo et al. 2010). The nitrocellulose membrane was incubated in Ponceau S solution (Sigma Aldrich) for 5 minutes, followed by a brief rinse in deionized water (destain) so that the bands were made visible. The membrane was then scanned in a GS-800 calibrated imaging densitometer (Bio-rad). After that, the membrane was extensively washed with 1x TBS-T and deionized water to remove staining.

3.3.3. Quantitative analysis

Quantity One Densitometry software (Bio-Rad) was used to quantify band intensities of the immunoblots.

IV. Results

4.1. Inflammatory biomarker profile in plasma samples

Neuroinflammatory process plays a key role in dementia and AD. As such many studies have addressed the potential of inflammatory biomarkers in AD diagnosis, however controversial findings have been reported. In an attempt to aid in the clarification of this issue, in this project we aimed to evaluate a panel of putative inflammatory biomarkers, in particular cytokines, in plasma samples, which is a biological fluid extensively used for the identifications of non-invasive peripheral biomarkers for AD. Samples were obtained from the CBC Cohort study and collected and selected as described in section 3.1 of methods.

Different inflammatory proteins were evaluated, 2 interleukines (IL-1β, IL-6) and 3 chemokines (IL-8, MCP-1, RANTES) in plasma samples by flow cytometry. These biomarkers were selected, based on the literature. Data were obtained for IL-6, IL-8, RANTES and MCP-1. Unexpectedly, IL-1β could not be detected in plasma samples, this could be related with sample storage at -80°C, potentially leading to IL-1β degradation. Therefore, for the purpose of this work, the biomarkers considered were IL-6, IL-8, MCP-1 and RANTES.

Plasma samples, included a total of 46 individuals that were subdivided into 3 groups according to the cognitive evaluation results (Section 3.2.1, Methods). The control group (both CRD- and MM-), a group with cognitive alteration (CRD+ and MM-), that putatively include patients with mild cognitive (MCI) impairment, and a group of individuals (CDR+/MM+), which includes 5 patients clinically diagnosed for AD type dementia. To determine if the study subgroups population follow a normal distribution (Gauss Curve) and since each group is constituted by less than 30 individuals, Shapiro-Wilk test was performed. Table 7 shows that subgroups do not follow a normal distribution thereby the subsequent statistical analyses were done using non-parametric tests (see flowchart, section 3.2.1 in methods).

Table 7 – Shapiro-Wilk test to check if the study population is normally distributed.

Test of Normality: Shapiro-Wilk

	Statistic	df	p
IL-6	0,23	39	0,00
IL-8	0,85	39	0,00
MCP-1	0,74	39	0,00
RANTES	0,51	39	0,00

As mentioned, the detection of cytokines levels was performed by flow cytometry. Statistical analysis of the data obtained revealed that there was no difference in concentrations among the groups for IL-6, or neither for RANTES and MCP-1, as shown in Table 8. However for the chemokine IL-8, a significant difference among groups was detected ($p < 0,05$, Kruskal-Wallis test).

Table 8 - Hypothesis test summary for the cytokines tested.

Hypothesis Test Summary			
Null Hypothesis	**Test**	**p**	**Decision**
The distribution of IL-6 is the same across categories of Groups.	Independent Samples Kruskal-Wallis	0,08	Retain the null hypothesis.
The distribution of MCP-1 is the same across categories of Groups.		0,89	Retain the null hypothesis.
The distribution of RANTES is the same across categories of Groups.		0,93	Retain the null hypothesis.
The distribution of IL-8 is the same across categories of Groups.		0,03	**Reject the null hypothesis.**

For IL-6 there were no statistically differences among the three study groups ($p=0,08$, Kruskal-Wallis test). Despite that box plot graphs (Figure 10) showed a slight increase of IL-6 levels from CDR+/MM- to CDR+/MM+ patients (possible AD type dementia) (0 vs 0,22 pg/mL, respectively). CDR+/MM- (Controls) did not differ from CDR+/MM- patients (both with 0 pg/mL).

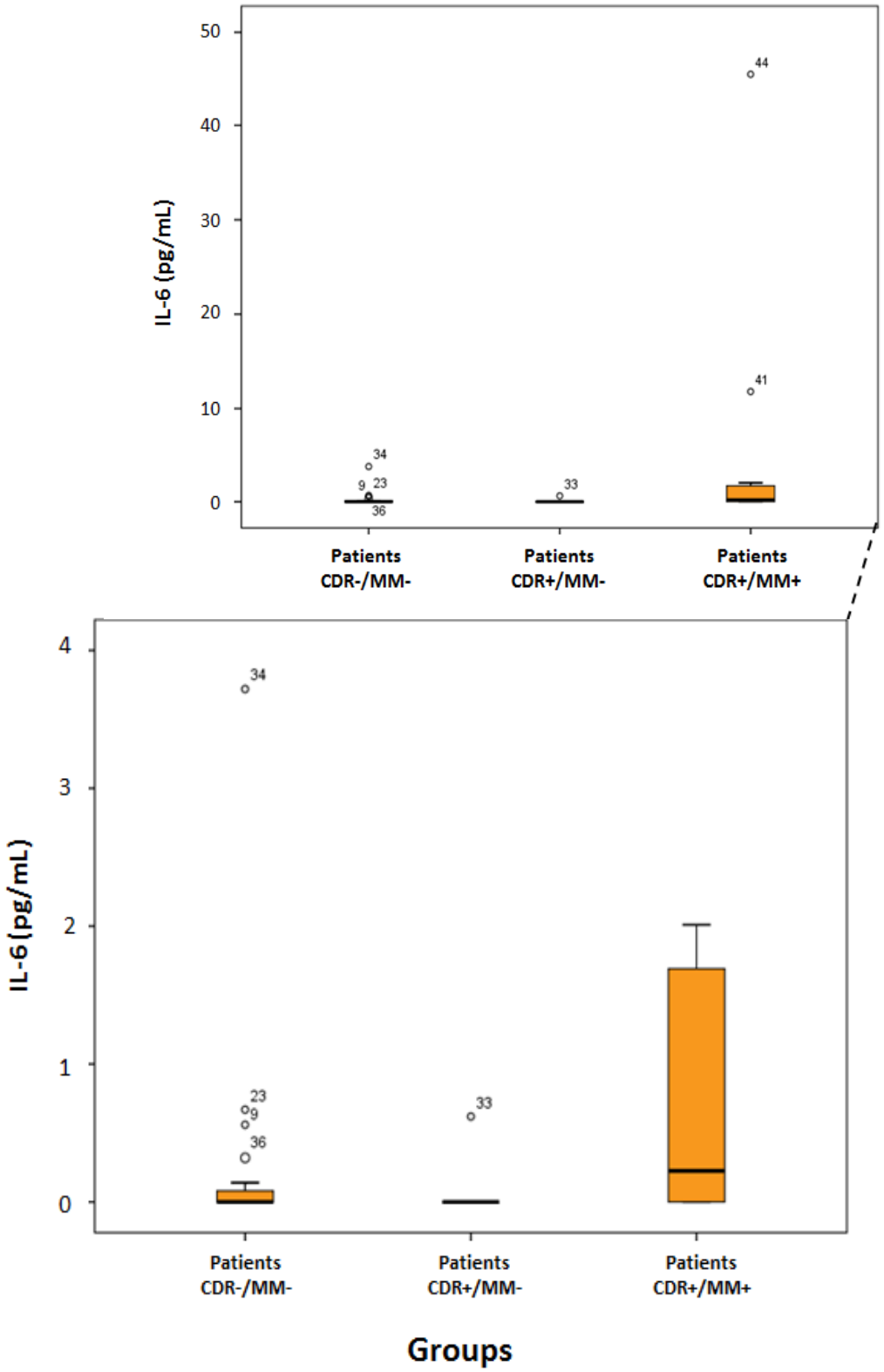
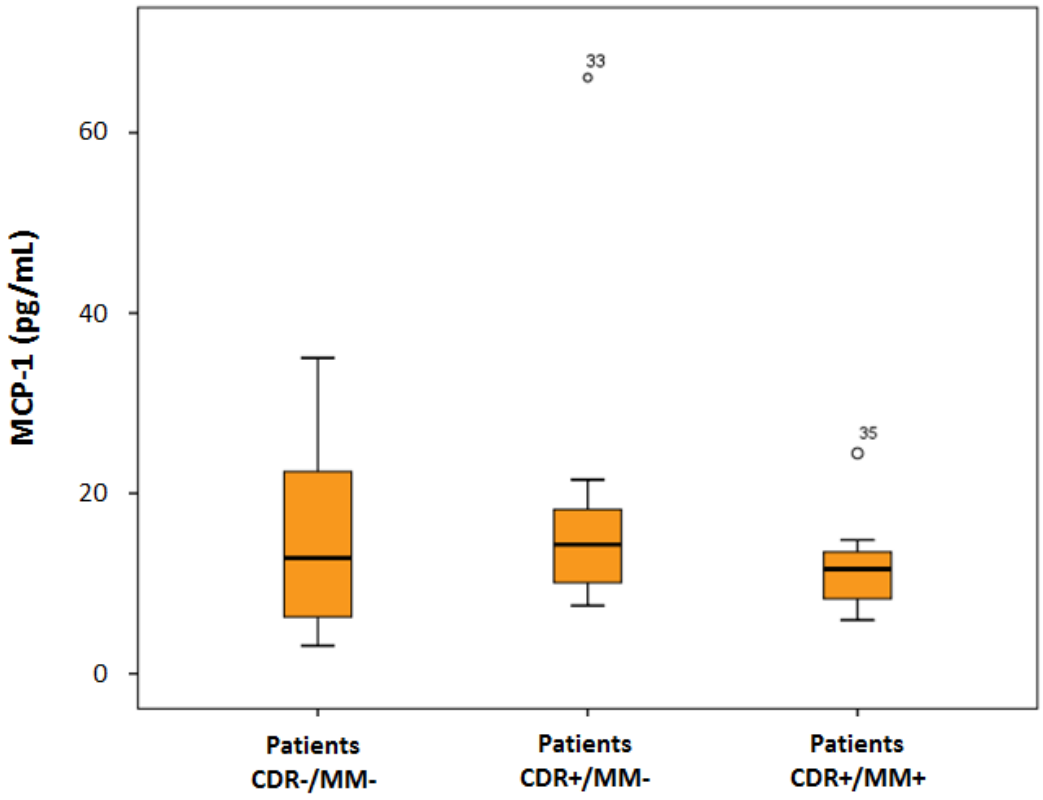


Figure 10 – **IL-6 concentrations for the three study groups.** Flow cytometry analysis allowed the determination of the absolute value of IL-6 analyte in plasma samples. Control group (n=25), CDR+/MM- (n=9) and CDR+/MM+ (n=12). The middle line in the box represents the median. The Kruskal-Wallis test was applied, p=0,08 and Mean Rank: CDR-/MM-= 22,32; CDR+/MM-= 19,11; CDR+/MM+= 29,25.

Flow cytometry data analysis for MCP-1 and RANTES (Figure 11) did not show statistically significant differences between groups (p=0,89 and p=0,93, respectively, Kruskal-Wallis test). MCP-1 exhibited a median concentration level of 11,61 pg/mL in the CDR+/MM+ group, of 14,62 pg/ml in CDR+/MM- group and of 12,82 pg/ml in the CDR-/MM- control individuals, showing a tendency to a slight increase from controls to putatively MCI patients (CDR+/MM-). RANTES showed a median concentration value of 6279,42 pg/ml vs 5871,61 pg/ml (CDR+/MM+ vs CDR-/MM-). Both chemokines presented two common outliers, #33 and #35, in the CDR+/MM- and CDR+/MM+ groups respectively, which could contribute to a higher median value (middle line) of the total group. An outlier (#23) was present in the CDR-/MM- group for RANTES and also for IL-6.

A.



B.

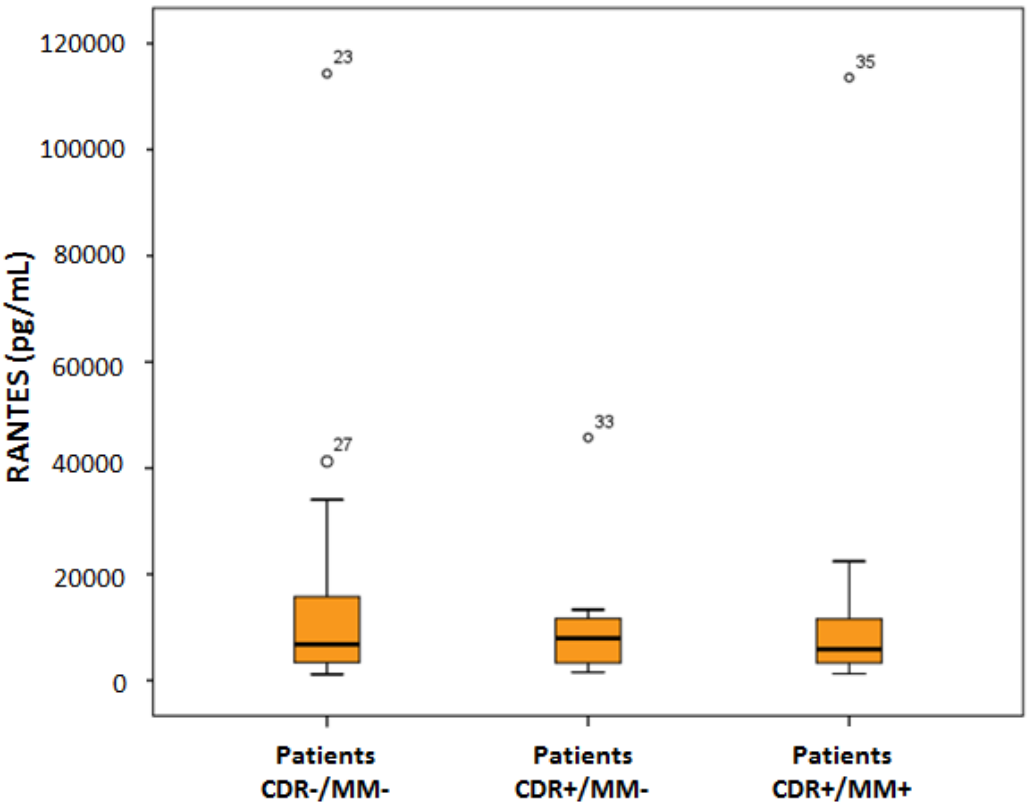


Figure 11 – MCP-1 and RANTES concentrations for the three study groups. Flow cytometry analysis allowed the determination of the absolute value of **(A)** MCP-1 and **(B)** RANTES analytes in plasma samples. The Kruskal-Wallis test was applied for both chemokines. For MCP-1, some values could not be determined, and as such control group (n=22), CDR+/MM- (n=7) and CDR+/MM+ (n=10). p=0,89 and Mean Rank: CDR-/MM-= 19,77; CDR+/MM-= 22,86; CDR+/MM+=18,5. For RANTES control group (n=25), CDR+/MM- (n=9) and CDR+/MM+ (n=12). p=0,93 an Mean Rank: CDR-/MM-= 24,16; CDR+/MM-= 23,11; CDR+/MM+= 22,42. The middle line in the box represents the median.

Noticeably, statistical differences were detected in the case of IL-8 chemokine (Figure 12), for which decreased levels were observed in both patients groups when compared to controls subjects. While control individuals (CDR-/MM-) exhibited an IL-8 concentration mean value of 1,43 pg/ml the possible AD group exhibited a mean value of 0,75 pg/ml ($p<0,05$, Kruskal Wallis test). The concentrations values among groups appear to be more consistent than for the other inflammatory markers tested, since only the CDR+/MM+ patients group exhibited one outlier (#44), also presented in IL-6, with higher IL-8 concentrations. This may also explain the slight increase in the median value of this group when compared to CDR+/MM- group (0,57 pg/ml). The outlier #33, was present in all the other inflammatory markers (Figure 10 and 11), except for IL-8.

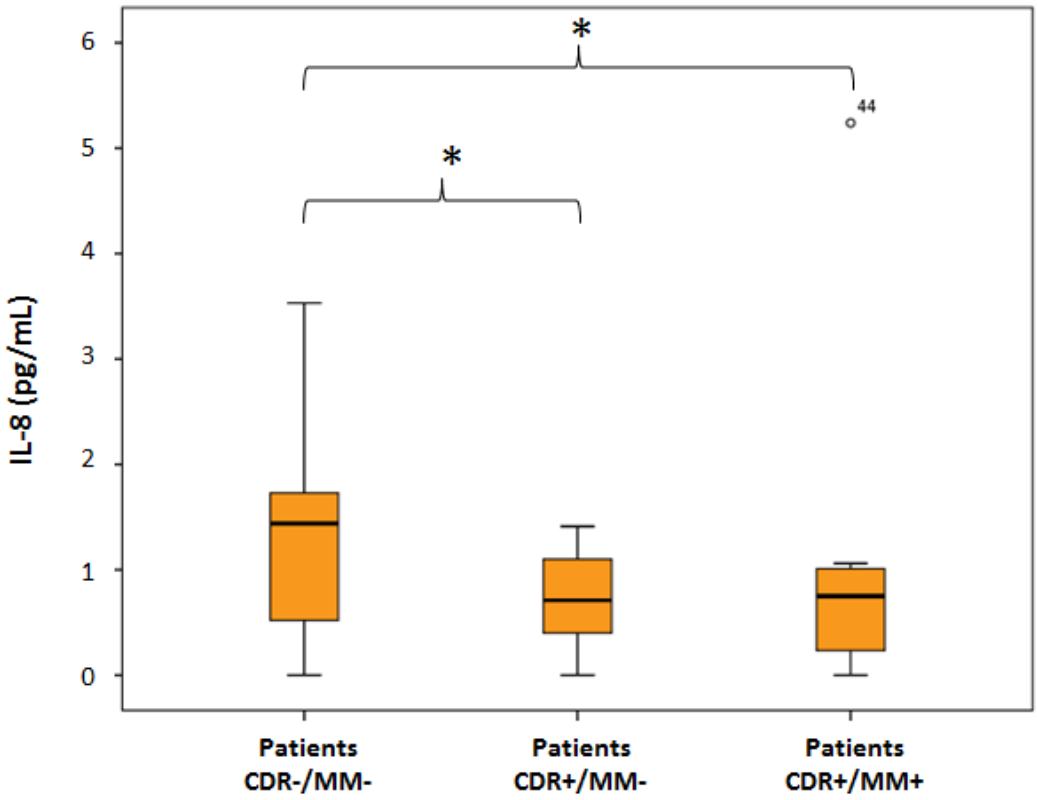


Figure 12 – IL-8 concentrations for the three study groups. Flow cytometry analysis allowed the determination of the absolute value of IL-6 analyte in plasma samples. Control group (n=25), CDR+/MM- (n=9) and CDR+/MM+ (n=12). The middle line in the box represents the median. The Kruskal-Wallis test was applied, p=0,03 and Mean Rank: CDR-/MM-= 28,32; CDR+/MM-= 17,33; CDR+/MM+= 18,08.

4.1.1. Paired analysis of the inflammatory biomarkers

The IL-8, MCP-1, RANTES and IL-6 concentrations obtained for the plasma samples of patients with cognitive alterations (PCA), either CDR+/MM- or CDR+/MM+, were paired with age- and sex-matched CDR-/MM- individuals (C, Control individuals) (n=21 pairs), as displayed in Table 9. A Wilcoxon Signed Rank test was used for statistical analysis and, consistently with the data previously presented, statistically significant differences were detected for the IL-8 inflammatory protein (p=0,02). None of the other inflammatory proteins were statistically different among the PCA and C groups.

Table 9 – Paired analysis of the cytokines in plasma samples. Concentrations of IL-8, MCP-1, RANTES and IL-6 in the plasma of patients with cognitive alterations (PCA) that include the CDR+/MM- and CDR+/MM+ patients and age- and sex- matched CDR-/MM- (C) (n=21 pairs).
p values are displayed as well (Wilcoxon Signed Rank Test).
N. D. – Not Determined

C nr.	PCA nr.	C IL-8 n=21 pg/mL	PCA IL-8 n=21 pg/mL	C MCP-1 n=18 pg/mL	PCA MCP-1 n=17 pg/mL	C RANTES n=21 pg/mL	PCA RANTES n=21 pg/mL	C IL-6 n=21 pg/mL	PCA IL-6 n=21 pg/mL
1	**22**	0,22	0,42	5,19	14,33	3486,16	10826,79	0,00	0,00
2	**23**	0,46	1,22	14,65	14,92	16140,80	1510,29	0,00	0,00
3	**24**	1,42	0,00	8,24	7,56	11079,45	11668,23	0,14	0,00
4	**25**	1,44	1,03	22,41	N. D.	41258,67	11432,98	0,00	0,00
5	**26**	1,56	0,00	14,15	8,00	2779,10	7936,35	0,00	0,00
6	**27**	3,53	0,99	12,85	9,50	9227,55	22442,50	0,56	0,00
7	**28**	1,99	0,36	32,94	13,35	9351,03	3013,26	0,13	0,45
8	**29**	2,22	0,63	14,49	N. D.	15781,65	7352,59	0,00	0,00
9	**30**	2,64	1,01	23,59	66,11	2854,28	45764,34	0,32	0,62
10	**31**	1,62	0,03	N. D.	8,30	34064,93	7111,73	3,72	0,59
11	**32**	1,39	0,11	N. D.	5,95	2166,80	1569,95	0,00	0,00
12	**33**	1,58	5,24	5,95	10,39	3088,88	3574,52	0,00	45,55
13	**34**	1,73	0,00	5,14	8,24	2327,29	4458,37	0,00	11,81
14	**35**	0,36	0,71	10,25	N. D.	12792,91	6779,46	0,00	0,00
15	**36**	0,49	0,87	10,96	24,44	27081,41	113577,00	0,00	2,01
16	**37**	1,44	0,91	13,50	13,50	5756,63	1269,16	0,00	0,00
17	**38**	1,42	0,44	6,31	14,84	3361,64	11696,21	0,00	1,37
18	**39**	0,00	1,10	N. D.	N. D.	13960,64	13312,11	0,00	0,00
19	**40**	0,52	0,40	12,82	21,52	4128,39	1913,89	0,00	0,00
20	**41**	2,09	1,41	28,46	12,18	114313,90	3296,53	0,67	0,00
21	**42**	1,91	1,06	35,02	12,82	16745,53	4631,49	0,00	0,00
Wilc.	***p***	**IL-8**	**0,02**	**MCP-1**	0,69	**RANTES**	0,43	**IL-6**	0,33

4.2. IL-8 correlations with CDR stages

Patients with cognitive decline were evaluated by the CDR and the MMSE tests. Correlation analysis of both tests with our inflammatory biomarker candidates revealed that only IL-8 concentrations correlated with CDR (Table 10, Spearman´s Correlation). A statistical significant ($p<0,01$) negative correlation was detected for CDR scores:IL-8 concentrations ($r=-0,38$, $p=0,009$).

Table 10 – Correlation between IL-8 and CDR scores by Spearman's Correlation.
** Correlation is significant at the 0,01 level (p*)*.

Correlations				
			IL-8	**CDR Stages**
Spearman's Correlation	**IL-8**	**Correlation Coefficient**	1	-0,38**
		P	-	0,009
		N	46	46
	CDR Stages	**Correlation Coefficient**	-0,38**	1
		P	0,009	-
		N	46	46

CDR is a five-point scale in which CDR-0 indicates no cognitive impairment, and the remaining four points refers to various stages of dementia (very mild or suspected dementia, mild, moderate and severe).

Kruskall-Wallis test was used for statistical analysis of IL-8 concentrations in the CDR groups independently. No significant differences were detected for IL-8 and MM. IL-8 was decreased in the 4 stage groups of CDR ($p<0,05$), as seen in Figure 13. A higher decrease in IL-8 concentrations could be observed for the mild dementia group, which could be related to a decrease number of subjects at this stage.

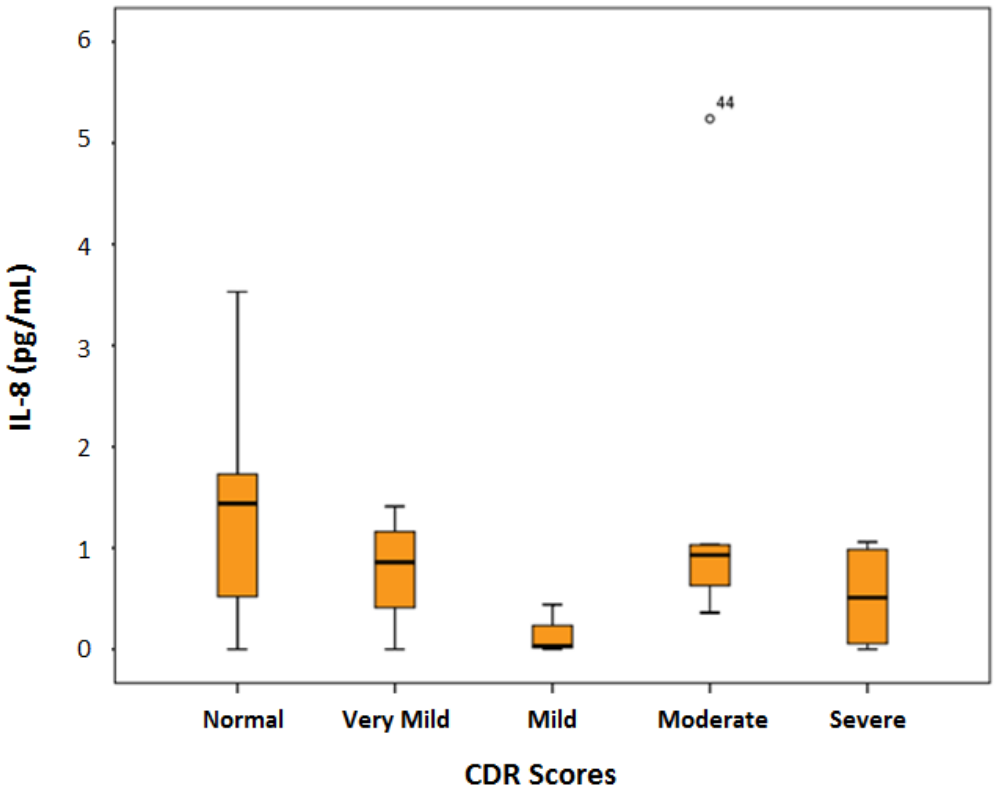


Figure 13 – IL-8 and CDR stages correlation. Box plot graph presenting the IL-8 levels in each score group of the CDR diagnosis exam by Kruskall-Wallis test showing statistically significant differences (p=0,009). Normal (n=25); Very Mild or Suspected (n=8); Mild (n=3); Moderate (n=6) and Severe (n=4).

4.3. Inflammatory proteins correlations

In order to evaluate if the cytokines correlate between them, the Spearman's Correlation was used. Data are presented in Table 11. Considering all subjects studied, a positive correlation, that was statistically significant (p<0,05), between the concentrations of IL-8 and MCP-1 (r= 0,32, p=0,03) and between IL-6 and MCP-1 (r= 0,31, p=0,04) could be observed. No significant correlations were identified for the other inflammatory protein concentrations (IL-8:IL-6 r=0,22, p=0,15; IL-8:RANTES r=0,07, p=0.64; MCP-1:RANTES r=0,22, p=0,15; RANTES:IL-6 r=0,26, P=0,08).

Table 11 – Correlations of the inflammatory proteins. Spearman's Correlation was used for correlation analysis. * Correlation is significant at the 0,05 level (p).

Correlations						
			IL-8	**MCP-1**	**RANTES**	**IL-6**
Spearman's Correlation	**IL-8**	**Correlation Coefficient**	1	0,32*	0,07	0,22
		P	-	0,03	0,64	0,15
		N	46	46	46	46
	MCP-1	**Correlation Coefficient**	0,32*	1	0,22	0,31*
		P	0,03	-	0,15	0,04
		N	46	46	46	46
	RANTES	**Correlation Coefficient**	0,07	0,22	1	0,26
		P	0,64	0,149	-	0,08
		N	46	46	46	46
	IL-6	**Correlation Coefficient**	0,22	0,31*	0,26	1
		P	0,15	0,04	0,08	-
		N	46	46	46	46

4.4. IL-8 effects on APP processing

IL-8 appears to be the most promising candidate inflammatory biomarker, as such its effects on APP were addressed. Few studies focused on the effects of cytokines on APP processing and Aβ production, and to our knowledge, little is known about IL-8 effects on APP processing. Therefore, preliminary experiments were carried to address this issue. Upon IL-8 incubation for 6h no differences could be detected for APP and sAPP secretion. Despite that at 24h, APP levels showed a tendency to increase in a dose-dependent manner, as shown in Figure 14. This was not accompanied by an increase on sAPP secretion (22C11, N-terminal APP antibody). In particular, for the longer incubation period and at the highest concentration (100 pg/mL), APP levels increased, by 0,7 fold when compared to non-IL-8 treated cells, as detected by the APP C-terminal antibody. Data suggest that IL-8 may impact on APP metabolism, but additional experiments need to be carried out.

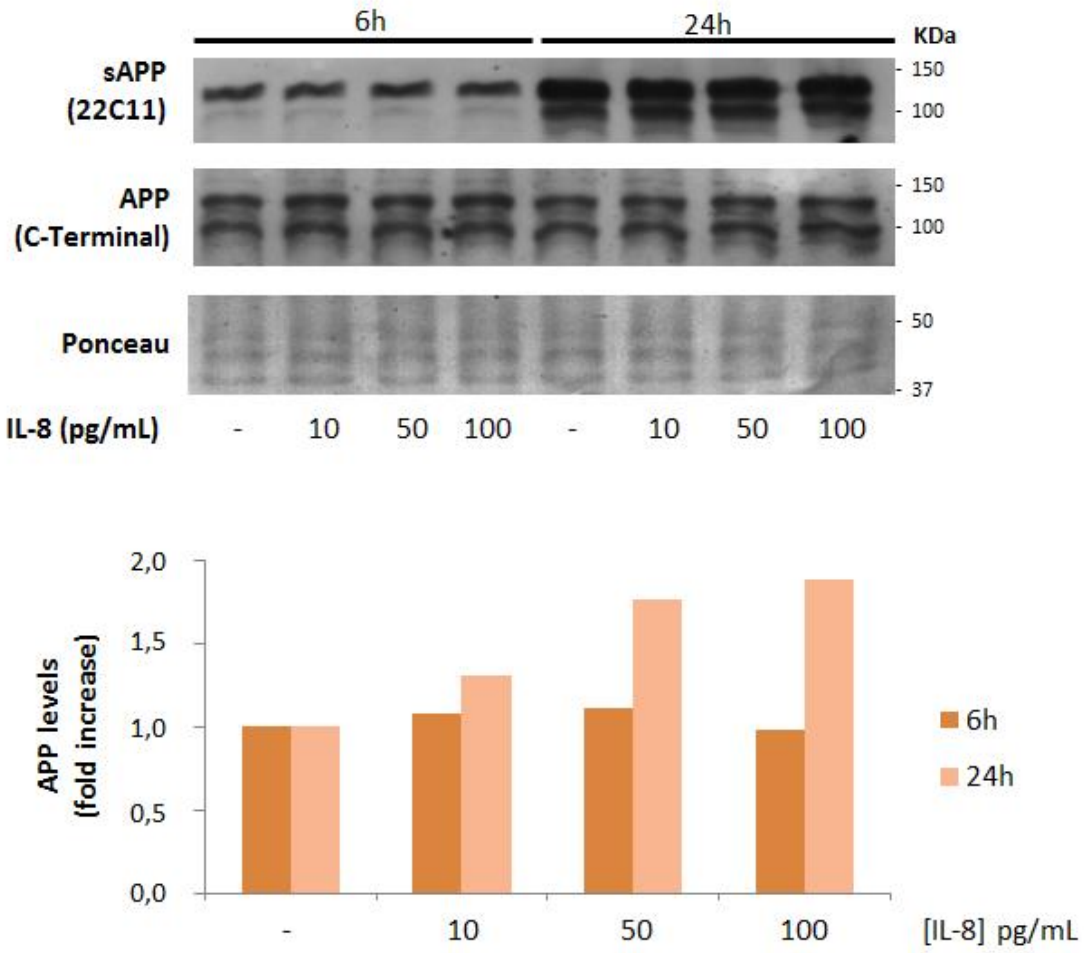


Figure 14 – IL-8 effects on APP processing. Upon IL-8 treatment for 6h and 24h, SH-SY5Y cells lysates and conditioned media were collected. Conditioned media were probed with the APP N-terminal 22C11 antibody and cell lysates with the APP C-Terminal antibody. Quantification of APP levels by densitometric scanning was normalized to protein levels determined by Ponceau S staining.

V. Discussion

Cognitive decline is a common feature of several neuropathologies, which complicates differential diagnosis of dementia subtypes. AD is one of the most common forms of dementia characterized by progressive cognitive decline, whose diagnosis requires a set of clinical and neurological examinations and evaluations to exclude other forms of dementia. Recently, a promising and accurate tool has emerged that test for a panel of CSF biomarkers, which together with the clinical examination, allows, distinction among neurodegenerative diseases, such as AD. However, CSF collection is an invasive procedure that requires a lumbar puncture, involves clinical doctors support and may have additional complications. Hence, many studies have addressed the potential of peripheral biomarkers in the diagnosis of AD, and other neuropathologies. Among the factors that may contribute to AD progression is neuroinflammation. Studies have focused on the potential of inflammatory biomarkers in AD diagnosis, however many of these data are still controversial. In this work we aimed to evaluate a panel of putative inflammatory biomarker candidates in plasma samples, of patients with cognitive decline, and with possible AD type dementia.

We evaluated different cytokines, including IL-6 and three chemokines (IL-8, MCP-1 and RANTES) in plasma samples of patients with cognitive alterations, which were identified by CDR and MM diagnostic tests. CDR is a numeric scale used to quantify the dementia severity symptoms that range from very mild (or suspected) to severe dementia stages. MM test also detects dementia, and several studies demonstrated that it is possible to distinguish some types of dementia with this diagnostic exam. A recent study showed lower points at baseline MM scores for AD patients comparatively to patients suffering from subtypes of frontotemporal lobar degeneration (FTLD) (Tan et al. 2013). The same work also showed that MM scores declined significantly faster in patients with AD than in the 2 FTLD subtypes analysed. Another study suggested that MM may be helpful in the differentiation of DLB and AD (Ala et al. 2002). In this sense, MM is a useful tool to detect dementia and cognitive alterations and putatively to aid in the identification of AD type dementia.

The panel of inflammatory proteins was evaluated in normal subjects, without cognitive dysfunction (the group designated as CDR-/MM-), in individuals with cognitive impairment that were negative for MM (designated as CDR+/MM-) and individuals with cognitive impairment that were positive for MMSE test (designated CDR+/MM+). The latest group included 5 patients that were already clinically diagnosed as AD patients. Most patients with MCI, from which 2 in 10 can progress to AD (Risacher et al. 2009; Kester et al. 2011; http://www.nia.nih.gov/alzheimers/publication/part-2-what-happens-brain-ad/changing-brain-ad), may present a normal MM test. Therefore our CDR+/MM- group can potentially include MCI patients.

In particular, IL-6 is a cytokine produced by neurons, microglia, astrocytes and endothelial cells, and it is up-regulated in CNS diseases where neuroinflammation has a key role (Erta et al. 2012). This pro-inflammatory protein is increased around SP in AD brains and IL-6 was shown to play a role in AD by increasing Aβ phagocytosis by microglia (Chakrabarty et al. 2010), supporting the notion that IL-6 could impact on Aβ clearance. IL-6 were reported to be increased in both CSF and plasma samples of AD patients (Hüll et al. 1996; Hampel et al. 2005; Licastro et al. 2000). Cojocaru et al. (2011) also reported an increase of IL-6 levels in serum of clinically diagnosed AD patients comparatively to controls. Studies suggested that elevated plasma levels of IL-6 increase the risk of developing dementia (Engelhart et al. 2004). In our work, for the majority of the samples, IL-6 levels were very low (around zero), although a tendency for an increase was observed for the CDR+/MM+ patients (0,22 pg/mL). Therefore, although our results were not statistically significant the tendency observed is in agreement with literature data. Nonetheless, it would be important to increase the number of samples in an attempt to better define the profile of this cytokine in AD patients. Moreover, and this is true for all cases, we cannot exclude that the methodology of sample collection and processing, the cognitive selection criteria used and that the interindividual variations may underlie and explain the differences between studies.

Among the chemokines analysed is RANTES, which is produced by astrocytes. RANTES, but also MCP-1 expression has been associated with a wide range of inflammatory disorders and some neurological diseases, including AD. According to Larlori et al. (2005) and Reale at al. (2012), higher levels of RANTES were evident in the plasma of AD compared to healthy subjects. However, like for many other cytokines, contradictory studies report that RANTES expression levels were lower in AD patients blood samples than in controls subjects (Kester et al. 2012). In our study group, no significant differences could be detected for the possible AD group (CDR+/MM+ group) when compared to the control individuals (CDR-/MM- group).

Other of the chemokines tested is MCP-1 that is produced by microglia and is able to stimulate astrocytes, participating in Aβ deposits elimination. Consistent data have been reported for this chemokine in AD. A significantly increase of MCP-1 levels in plasma and serum of MCI and mild AD patients but not in severe AD cases, when compared to controls have been reported (Galimberti, Fenoglio, et al. 2006; Kim et al. 2011), suggesting that MCP-1 plasma levels could be a useful biomarker to monitor the inflammatory process in AD. Similarly, according to Reale et al. (2012), lower plasma MCP-1 levels were detected in AD patients when compared with control individuals (Reale et al. 2012). However, in the present work, despite the slight decrease in the possible AD (CDR+/MM+) group, and the slight increase in the group that putatively include MCI patients (CDR+/MM-), comparatively to control individuals (CDR-/MM-), no statistically significant differences could be observed among the 3 groups.

As the case for IL-6, increasing the number of subjects could clarify the cognitive decline associated profile of this chemokine.

For all the above mentioned proteins, some outliers could be detected. These divergent values correspond to patients that presented levels higher or lower protein than median (for this case 3 standards deviations), which are automatically calculated and identified by the statistical program. Nonetheless, these values were not excluded from the median values calculated for each group. We analyzed each case individually. The outlier #23 is a control (CDR-/MM-) subject, with no cognitive dysfunction or other pathology reported; however, it was found altered for IL-6 and RANTES. This apparently healthy individual may have an unknown dysfunction or take some medication that leads to the changes observed in these cytokine levels. Another outlier that appears in these inflammatory proteins is #33, classified as a CDR+/MM- patient. This individual has several diseases including psychiatric disturbances. Neurological alterations, others than cognitive ones, as depression, can change the inflammatory components levels (Raison and Miller 2011; Najjar et al. 2013). The #35 outlier belongs to the CDR+/MM+ group and showed higher concentration levels for MCP-1 and RANTES chemokines. Similarly to #33, this outlier also presents psychiatric disorders among other diseases (arterial hypertension, dyslipidemia, gastric disease, osteoarticular disease), which can affect chemokines concentrations. Of note, our study group excluded subjects positive for the GDS scale, however, we did not exclude patients that were undertaking medication for depression or other psychiatric disorders, and that can now be negative for GDS scale.

Interestingly, significant differences arise for the IL-8 chemokine, which has been shown to have a protective role against Aβ-induced neurotoxicity (Ashutosh et al. 2011). However, many reports on IL-8 chemokine levels in MCI or AD are controversial. Despite that, previous studies mentioned that the circulating plasma IL-8 levels, were higher in controls than in MCI and AD patients (Kim et al. 2011). Our results were in agreement these findings showing a decrease of IL-8 concentration between both patients groups (CDR+/MM- and CDR+/MM+) and the control group (CDR-/MM-), statistically significant differences ($p<0,05$) according to Kruskal-wallis test. Of note, despite the presence of some common outliers for the other inflammatory biomarkers tested, only one outlier subject (#44) could be detected for IL-8. This suggests that this chemokine could be more specific for dementia than the other inflammatory proteins since it was not so affected by other pathologic conditions.

Additionally, when control subjects (CDR-/MM-) were randomly sex- and age-matched with cognitive decline patients, significant differences were also detected for IL-8, independently of the patient cognitive decline stage ($p=0,02$, Wilcoxon test). Taken together data suggest that IL-8 could be a putative biomarker candidate for cognitive decline, decreasing not only in CDR+/MM+ group (possible AD type dementia) but also in CDR+/MM-group (that may include MCI individuals and represent initial stages of dementia).

A relation between IL-8 and the CDR scores was also observed. IL-8 concentrations significantly decrease with the different stages of cognitive decline when compared to normal individuals. In our study group, these IL-8 concentrations did not change consistently along dementia progression scores. However, once again, we should take in consideration the number of subjects in each scored group. Hence, it would be relevant to repeat this study with an increased number of subjects.

Additionally, correlation analysis by Spearman's test showed a significant correlation ($p<0,05$) between the concentrations of IL-6 and MCP-1 ($r=0,31$, $p=0,04$) and the concentrations of IL-8 and MCP-1 ($r=0,32$, $p=0,03$). Although not directly for AD, several data supports the interplay between these proteins and the correlations observed among them. Previous studies showed correlations between the MCP-1 mRNA levels and other cytokines mRNA levels, including IL-6, in rats autoimmune myocarditis (Kobayashi et al. 2002). Further, it was also reported that IL-6 stimulation induces higher expression of MCP-1 mRNA in a human myeloma cell line (Arendt et al. 2002) and that MCP-1 can stimulate markers of inflammatory activation such as IL-6 secretion (Viedt et al. 2002). MCP-1 and IL-8, both chemokines type inflammatory proteins, have common functions, e.g. in the neuroinflammation process (Gerszten et al. 1999), what may somehow support these correlations, as they can both be stimulated during that process. Moreover, it was also showed that these chemokines positively correlated and can serve as good indicators of local inflammation in pathologies such as rheumatoid arthritis (Slavic, Stankovic, and Kamenov 2005) and also in acute inflammation induced by prolonged exercise (Skenderi et al. 2010). Both MCP-1 and IL-8 levels correlated positively with age, and significantly increased in CSF of MCI and AD patients (Galimberti et al. 2006). Presently, it is difficult to interpret the significance of these correlations and additional studies in larger samples are needed to understand the importance of those associations in cognitive decline and AD pathology.

As IL-8 appears to be the most promising candidate, experiments were also carried out to evaluate IL-8 effects on APP processing. Preliminary results showed that upon cell incubation with IL-8, there was a slight increase in the intracellular APP levels (APP not cleaved as detected by the APP C-terminal antibody), at the highest concentration and for the longer incubation period. Additional experiments should be directed for longer IL-8 exposure periods and also for the evaluation of other fragments resulting from APP cleavage, as sAPPα/β and Aβ itself. The study of IL-8 effects on APP will help in the understanding of the function and involvement of this chemokine in dementia, in particular in AD neuroinflammation process and disease progression.

Conclusion & Future Perspectives

In sum, cytokines and chemokines are important in triggering the immune response in CNS, being responsible for microglia and astrocytes activation and for recruitment of peripheral immune cells, respectively, to the site of Aβ deposition defining the extension of local inflammation. Hence, the release of these inflammatory agents may constitute potential biomarker candidates for AD diagnosis. The data herein presented suggests that IL-8 is the most promising peripheral inflammatory biomarker, useful for cognitive decline and AD detection. However, further studies will be needed to validate the results, particularly by increasing the number of subjects in the study group and also to include individuals with other neuropathological diseases, to address if IL-8 can be specific or not for dementia and/or AD. Of note, as AD is a multifactorial disorder it is expected that a panel of biomarkers, rather than a single biomarker, would be necessary to aid in a more specific and reliable disease diagnosis.

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VII. Annexes

In this section are indicated the equipment and composition of solutions used for the different techniques applied.

4.1. Flow Cytometry

Equipment:

- Accuri C6 Flow Cytometer (BD Biosciences)

Reagents/Solutions:

- CBA kit (BD Biosciences)

4.2. Cell culture

Equipment:

- Hera cell CO_2 incubator (Heraeus)
- Safety cabinet Hera safe (Heraeus)
- Inverted optical microscope (LEICA)
- Hemacytometer (Sigma)
- Sonicator (U200S IKA)
- Bath SBB6 (Grant)

Reagents/Solutions:

- Complete medium 10% FBS MEM:F12 (1:1)

- MEM (Gibco, Invitrogen)	4,805 g
- F12 (Gibco, Invitrogen)	5,315 g
- $NaHCO_3$ (Sigma)	1,5 g
- Sodium Pyruvate (Sigma)	0,055 g
- 1% Antibiotic/Antimycotic (AA) mix (Gibco, Invitrogen)	10 mL
- 10% FBS (Gibco, Invitrogen)	100 mL
- L-Glutamine (200mM stock solution)	2,5 mL

Dissolve in dH_2O and adjust to pH 7,4 and to a final volume of 1000 mL in dH_2O. Sterilize by filtering through a 0,2 µm filter and store at 4^{o}C.

- PBS (1x)
  For a final volume of 500 mL, dissolve one pack of BupH Modified Dulbecco's Phosphatase Buffered Saline Pack (Pierce) in deionized H_2O. Final composition:
  - 8 mM Sodium Phosphatase
  - 2 mM Potassium Phosphatase
  - 140 mM Sodium Chloride
  - 10 mM Potassium Chloride

  Sterilize by filtering through a 0,2 µm filter and store at 4^oC

- RIPA buffer (Sigma-Aldrich)
  To 6,5 mL of RIPA buffer add:
  - 40,3 µL NaF
  - 65 µL NaO
  - 65 µL Protease inhibitor cocktail

4.3. Protein content determination

Equipment:

- Infinite M200 (Tecan) and I-control™ software

Reagents/Solutions:

- BCA assay kit (Pierce, Rockford, IL)
- Bovine serum albumin (BSA) (Pierce)
- Working reagent (50 Reagent A : 1 Reagent B)
  - Reagent A: sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,2 N sodium hydroxide.
  - Reagent B: 4% cupric sulfate.

4.4. SDS-PAGE

Equipment:

- Electrophoresis system (Hoefer SE600 vertical unit)
- Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotec)

Reagents/Solutions:

- Acrylamide stock mixture (30% acrylamide, 0,8% Bisacrylamide)
  To a 70 mL of deionized H_2O add:
  - 29,2 g Acrylamide
  - 0,8 g Bisacrylamide

  Mix until the solute has dissolved. Adjust the volume to 100 mL with deionized water. Filter through a 0,2 µm filter and store at 4°C.

- Stacking gel and resolving gel

	Stacking gel	Resolving gel
	3,5%	**7,5%**
H_2O	13,2 mL	29,63 mL
Acrylamide stock mixture	2,4 mL	15 mL
UGB (5x)	4,0 mL	--
LGB (4x)	--	15 mL
10% APS	200 µL	300 µL
10% SDS	200 µL	--
TEMED	20 µL	30 µL

- UGB (Upper gel buffer) (5x)
  To 900 mL of deionized H_2O add:
  - 75,69 g Tris

  Mix until the solute has dissolved. Adjust the pH to 6,8 and adjust the volume to 1 L with deionized H_2O.

- LGB (Lower gel buffer) (4x)
  To 900 mL of deionized H_2O add:
  - 181,65 g Tris
  - 4 g SDS

  Mix until the solutes have dissolved. Adjust the pH to 6,8 and adjust the volume to 1 L with deionized H_2O.

- APS (Ammonium Persulfate) 10%
  In 10 mL of deionized H_2O dissolve 1 g of APS. Note: prepare fresh before use.

- SDS (Sodium dodecilsulfate) 10 %
  In 10 mL of deionized H_2O dissolve 1 g of SDS.

- Loading gel buffer (4x)
  - 2,5 mL (250 mM) Tris solution (pH 6,8) 1 mM
  - 0,8 g (8%) SDS
  - 4 mL (40%) Glicerol
  - 2 mL (2%) Beta-Mercaptoetanol
  - 1 mg (0,01%) Bromofenol blue

  Adjust the volume to 10 mL with deionized H_2O. Store in darkness at room temperature.

- Tris 1 M (pH 6,8) solution
  To 150 mL of deionized H_2O add 30,3 g Tris base. Adjust the pH to 6,8 and adjust the final volume to 250 mL.

- 10x Running buffer
  - 30,3 g (250 mM) Tris
  - 144,2 g (2,5 mM) Glycine
  - 10 g (1%) SDS

  Dissolve in deionized H_2O, adjust the pH to 8,3 and adjust the volume to 1 L.

4.5. Western-Blotting

Equipment:

- Transphor Electrophoresis unit (Hofer TE 42)
- Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotec)

Reagents/Solutions:

- 1x Transfer buffer
  - 3,03 g (25 mM) Tris
  - 14,41 g (192 mM) Glycine

Mix until the solutes have dissolved. Adjust pH to 8,3 with HCl and adjust the volume to 800 mL with deionized H_2O. Just prior to use add 200 mL of methanol (20%).

4.6. Immunoblotting

- 10x TBS (Tris buffered saline)
  - 12,11 g (10 mM) Tris
  - 87,66 g (150 mM) NaCl

  Adjust the pH to 8,0 with HCl and adjust the volume to 1 L with deionized H_2O.

- 10x TBS-T (TBS + Tween)
  - 12,11 g (10 mM) Tris
  - 87,66 g (150 mM) NaCl
  - 5 mL (0,05%) Tween 20

  Adjust the pH to 8,0 with HCl and adjust the volume to 1 L with deionized H_2O.

- Ponceau S solution

Dissolve 0,1 g of Ponceau S (Sigma) in 100ml of 5% acetic acid solution (5 mL of acetic acid dissolved in 95 mL of deionized H_2O).

- Blocking solution

  5% of BSA (Bovine serum albumin, NZytech) in 1x TBS-T.

- ECL Solutions

- Luminata Crescendo (Millipore)
- Home-made ECL:
  - Solution A – ECL Luminol Solution (Stock Solution)
    - 20 mM Luminol (in DMSO)* 1.25 mL (100 $M)
    - 100 mM 4-iodophenol (in DMSO)* 5 mL (2mM)
    - 0.1 M Tris (pH 9.35) 125 mL (50 mM)

    Adjust volume to 250 mL with dH2O. * Protect from the light.
  - Solution B – Hydrogen Peroxide

- Developer and fixer solution (Sigma)

- Membrane stripping solution
  - 3,76 g (62,5 mM) Tris-HCl (pH 6,7)
  - 10 g (2%) SDS
  - 3,5 mL (100 mM) Beta-mercaptoetanol)

  Dissolve Tris and SDS in deionized H_2O and adjust with to pH 6,7. Add the mercaptoetanol and adjust volume to 500 mL.

4.7. Quantitative analysis

Equipment:

- GS-710 calibrated imaging densitometer (Bio-Rad).