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Catarina de Barros Pinto Salvador Domingues

Impacto das Quimiocinas na Doença de Alzheimer

Chemokines Impact in Alzheimer's Disease

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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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Por todo o apoio, compreensão e força, dedico esta dissertação aos meus pais, irmãos e sobrinho Guilherme.

o júri

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palavras-chave

Doença de Alzheimer, Neuroinflamação, Quimiocinas, Proteína Percursora Amilóide, Tau, Apoptose.

resumo

A Doença de Alzheimer (DA) é uma doença neurodegenerativa, caracterizada pela presença de placas senis extracelulares, tranças neurofibrilares intracelulares e perda sináptica.

A neuroinflamação tem sido associada com algumas doenças neurodegenerativas, tal como a DA. Na DA, a produção e agregação aumentada do péptido Aβ, tem um papel fundamental na activação do processo inflamatório, que pode ser importante nas fases iniciais da doença, devido à remoção de Aβ e à proteção do cérebro. No entanto, uma inflamação crónica leva a um aumento de mediadores inflamatórios como são as citocinas, libertadas por microglia activada, astrócitos e neurónios. A produção excessiva de componentes inflamatórios promove alterações tanto na expressão como no processamento da proteína percursora amilóide (APP), levando a uma maior acumulação de Aβ e fosforilação anormal da proteína tau. Isto resulta em efeitos neurotóxicos, dano irreversível e perda neuronal.

A inflamação crónica é uma característica da DA, no entanto pouco se sabe sobre os efeitos de algumas quimiocinas na sua patogénese. Assim, o principal objectivo desta tese foi o estudo do impacto da IL-8 e da MCP-1 na apoptose, APP e tau. Ambas as quimiocinas em estudo resultaram em pequenas alterações ao nível da citotoxicidade de células SH-SY5Y diferenciadas, tendo sido apenas observado um aumento significativo da apoptose para MCP-1 à concentração mais elevada. Relativamente ao processamento de APP, não foram observadas alterações significativas, no entanto alguma tendência para aumentar a diferentes concentrações e períodos foi obtida tanto para a IL-8 como para a MCP-1. Ao nível da tau e outras proteínas associadas ao citoesqueleto, foi possível observar uma tendência de aumento do resíduo fosforilado Ser396 às concentrações mais elevadas assim como alterações na actina e tubulina, com um aumento da α-tubulina acetilada. Este efeito pode ser traduzido em alterações na arquitetura e sobrevivência neuronal. Assim sendo, estudos adicionais podem contribuir para uma melhor compreensão do modo de ação destas quimiocinas na patogénese da DA.

keywords

Alzheimer's Disease, Neuroinflammation, Chemokines, Amyloid Precursor Protein, Tau, Apoptosis.

abstract

Alzheimer's Disease (AD) is a neurodegenerative disorder neuropathologically characterized by the presence of extracellular senile plaques, intracellular neurofibrillary tangles and synaptic loss.

Neuroinflammation has been associated with some neurodegenerative diseases, such as AD. In AD, increased Aβ production and aggregation, have a fundamental role in the activation of the inflammatory process. In turn, this could be fundamental in the early stages of this pathology, regarding the Aβ clearance and brain protection. However, chronic inflammation leads to an increase of the inflammatory mediators, such as cytokines, released by activated microglia, astrocytes, and neurons. The excessive production of these inflammatory components promotes alterations in both amyloid precursor protein (APP) expression and processing, stimulating the increase of Aβ accumulation and abnormal tau phosphorylation. This results in neurotoxic effects, irreversible damage and neuronal loss.

Chronic inflammation is a feature of AD however, little is known about the effects of some chemokines on its pathogenesis. Thus, the main aim of this thesis was to study the impact of the interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) on apoptosis, APP and tau. The both studied chemokines resulted in small alterations regarding the cytotoxicity on SH-SY5Y differentiated cells, being a significant increase in apoptosis observed only for the MCP-1 at the highest concentration. For the APP processing no significant differences were obtained, although a tendency to increase at different concentrations and periods was registered for both IL-8 and MCP-1. With respect to tau and other cytoskeleton-associated proteins, it was possible to observe a tendency to increase in the phosphorylated residue (Ser396) at the higher concentrations, as well as alterations on actin and tubulin with an increase on acetylated-α tubulin. This effect can be translated by neuronal architectural and survival alterations. Therefore additional studies could contribute to a better understanding of the way that these chemokines act on AD pathogenesis.

List of contents

List of abbreviations

A	ACH	Amyloid Cascade Hypothesis
	AD	Alzheimer's Disease
	AICD	APP Intracellular Domain
	ALS	Amyotrophic Lateral Sclerosis
	APLP1/2	Amyloid Precursor Like Protein 1/2
	ApoE	Apolipoprotein E
	APP	Amyloid Precursor Protein
	APS	Ammonium Persulfate
	Aβ	β-Amyloid peptide
B	BACE1	β-site APP-Cleaving Enzyme 1
	BBB	Blood Brain Barrier
	BCA	Bicinchoninic Acid
	Bcl2	B-cell lymphoma 2
	Bim	Bcl-2-interacting mediator
	BSA	Bovine Serum Albumin
C	C83	C-terminal fragment with 83 amino acids
	C99	C-terminal fragment with 99 amino acids
	Cdk5	Cyclindependent Kinase 5
	CDR	Clinical Dementia Rating
	C_f	Final Concentration
	CRP	C-Reactive Protein
	CSF	Cerebrospinal Fluid
	CTF	C-Terminal Fragment
D	DAPI	4',6-diamidino-2-phenylindole
	DMSO	Dimethyl Sulfoxide
	DNA	Deoxyribonucleic Acid
E	ECL	Enhanced Chemioluminescence
	EO-FAD	Early-Onset Familial Alzheimer's Disease
F	FBS	Fetal Bovine Serum
	FDG	18F-2-deoxy-2-fluoro-D-glucose
G	GSK-3β	Glycogen Synthase Kinase-3β
H	HD	Huntington's Disease
	HRP	Horseradish Peroxidase
I	IFN	Interferon

	IL	Interleukin
	IL-1ra	Interleukin-1 Receptor Antagonist
L	LB	Loading Gel Buffer
	LGB	Lower Gel Buffer
	LOAD	Late-Onset Alzheimer's disease
M	MAP-2	Microtubule-Associated Protein 2
	MCI	Mild Cognitive Impairment
	MCP-1	Monocyte Chemoattractant Protein-1
	MEM	Minimal Essential Medium
	MIP-1	Macrophage Inflammatory Protein-1
	MMSE	Mini-Mental State Examination
	MRI	Magnetic Resonance Imaging
	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	NaF	Sodium Fluoride
	NaO	Sodium Orthovanadate
	ND	Neurodegenerative Diseases
	NFT	Neurofibrillary Tangles
	NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association
	NK cells	Natural Killer cells
	NO	Nitric Oxide
O	ON	Overnight
P	PBS	Phosphate Buffered Saline
	PD	Parkinson's Disease
	PET	Positron Emission Tomography
	PFA	Paraformaldehyde
	PIB	Pittsburgh compound B (2-[4´-(methylamino)phenyl]-6-hydrobenzothiazole)
	PSEN	Presenilin
	p-tau	Hyperphosphorylated Tau
R	Rac	Ras-related C3 Botulinum Toxin Substrate
	RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
	rCMRGLc	Regional Cerebral Metabolic Rate for Glucose
	ROS	Reactive Oxygen Species
	RT	Room Temperature

S	sAPP	Soluble Fragment APP
	SDS	Sodium Dodecyl Sulfate
	SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
	SP	Senile Plaques
T	TBS-T	Tris-Buffered Saline - Tween 20
	TEMED	Tetramethylethylenediamine
	TGF	Transforming Growth Factor
	Th1 cells	Helper T cells
	TNF	Tumor Necrosis Factor
	t-tau	Total Tau
U	UGB	Upper Gel Buffer
V	VaD	Vascular Dementia

1 – Introduction

1 – Introduction

1.1 – Neurodegenerative Diseases

Neurodegenerative diseases (ND) are multifactorial debilitating disorders of the nervous system that affect approximately 30 million individuals worldwide. Alzheimer's (AD), Parkinson's (PD), Huntington's (HD) diseases and amyotrophic lateral sclerosis (ALS) are a ND group characterized by separate etiologies with distinct morphological and pathophysiological features. Abnormal protein dynamics with defective protein degradation and aggregation; oxidative stress and free radical formation like the reactive oxygen species (ROS); impaired bioenergetics and mitochondrial dysfunction as well as exposure to metal toxicity, are among the etiology factors of ND (Figure 1) and also strongly associated with ageing [1].

All these factors will contribute to the neurodegenerative process by a slow and progressive dysfunction and loss of neurons in the central nervous system, that can ultimately lead to dementia [2].

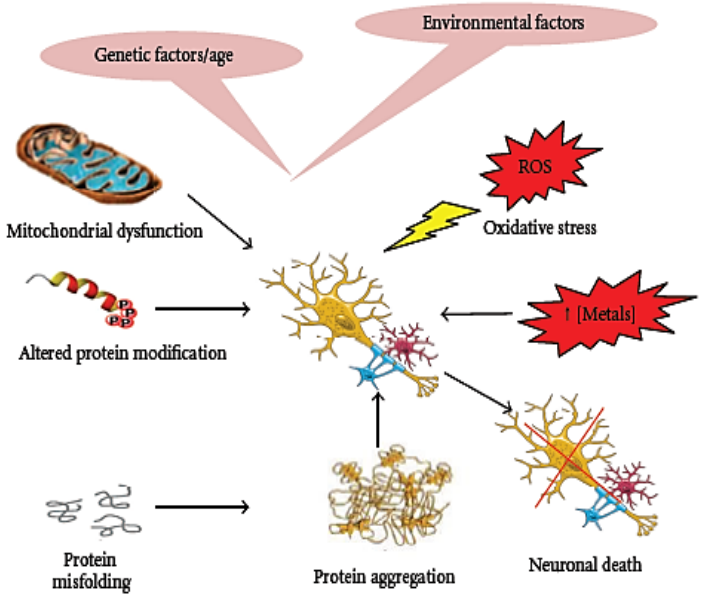

Figure 1 – Factors associated with neurodegenerative diseases. Genetic factors/age and environmental factors as part of the etiology of neurodegenerative diseases (adapted from [1]).

Dementia is an overall term for diseases and conditions characterized by a decline in memory or other thinking skills, that affects a person's ability to perform everyday activities [3]. There are many different types of dementia although some are far more common than others. Alzheimer's disease is the most common type of dementia, corresponding to about 60-70% of the cases, while vascular dementia (VaD) is the second, with about 20% of all cases [4–6].

Studies based on epidemiological data acquired over recent years suggested that 24.2 million people live with dementia and 4.6 million new cases arise every year. The prevalence rates for Alzheimer's disease also rise exponentially with age, increasing markedly after 65 years old. Given that both established and developing nations are rapidly aging, the frequency is expected to double every 20 years until 2040 [7].

1.2 – Alzheimer's Disease (AD)

In 1907, the German psychiatrist and neuropathologist Alois Alzheimer described the case of a woman who presented a relatively rapidly deteriorating memory along with psychiatric disturbances. AD is today, as it was then, a relentless neurologic deterioration (Figure 2) accompanied by a massive neuronal loss [5]. It is characterized by progressive cognitive decline usually beginning with impairment in the ability to form recent memories, but inevitably affecting all intellectual functions, leading to complete dependence for basic functions of daily life and premature death [7]. Behavioral and psychiatric symptoms, clustered into agitation/ aggression, mood disorders, and psychosis, may occur along the disease's progression [8].

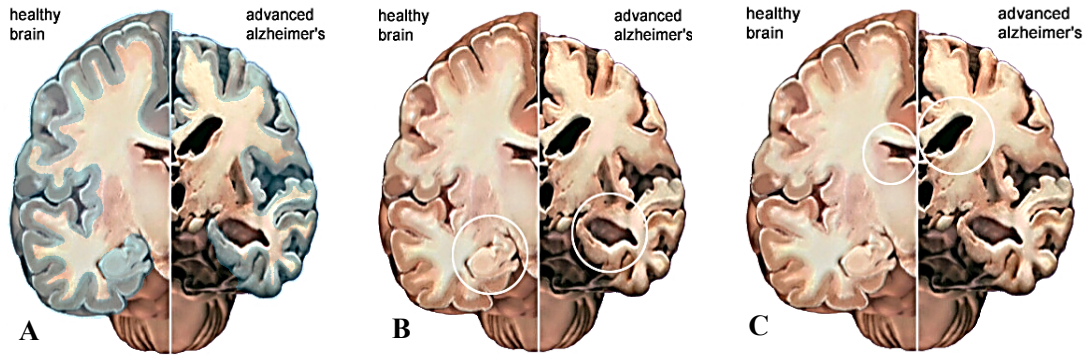

Figure 2 – Massive cell loss in the Alzheimer's brain. (A) The cortex (*blue contour*) shrivels up, damaging areas involved in thinking, planning and remembering; **(B)** Shrinkage is especially severe in the hippocampus (*white circles*), an area of the cortex that plays a key role in the formation of new memories and **(C)** Ventricles *(marked by the white circles*) that are fluid-filled spaces within the brain, grow larger (adapted from http://www.alz.org/braintour).

1.2.1 – Etiology of AD

Even though the etiological mechanisms underlying the neuropathological changes in Alzheimer's disease (AD) remain unclear, there are a large number of factors that has been associated with the increased risk of AD, namely age, genetic and environmental factors [7].

- **Age**

Age represents, by far, the single greatest risk factor in the etiology of AD. Indeed, in genetically-predisposed individuals, the disease occurs around the age of 50. Therefore, regardless of whether one is genetically predisposed or not, aging is an essential factor in AD, strongly suggesting that an age-related process is involved in the development of the disease [5]. The incidence of AD increases with age and the majority of the individuals with the disease are at age 65 or older. In terms of the likelihood for developing Alzheimer's, it doubles about every five years after the age of 65 and above 85 the risk reaches nearly 50 percent [7].

- **Family History**

Following advanced age, family history is the second strongest risk factor for Alzheimer's disease. AD is considered to be a genetically dichotomous disease present in two forms: early-onset familial cases, EO-FAD (< 65 years old), usually characterized by Mendelian inheritance, and the most common form of the disease defined as late-onset, LOAD (> 65 years old), with no consistent mode of transmission. The "sporadic" AD is strongly influenced by genetic variants combined with life exposure factors, while EO-FAD is mainly caused by rare, fully penetrant mutations in three different genes, APP and Presenilins (listed in the Table I). Therefore those who have a parent, brother, sister or children with Alzheimer's are more likely to develop the disease, with an additional increased risk if more than one family member has the illness. When diseases tend to run in families, either heredity (genetics) or environmental factors, or both, may play a key role [9].

- **Genetics (heredity)**

Rare mutations in APP (Amyloid Precursor Protein), PSEN1 (Presenilin 1), and PSEN2 (Presenilin 2) virtually guarantee EO-FAD, which represents approximately 5% of the Alzheimer's disease patients. To date, 24 mutations have been reported for APP, 185 for

PSEN1, and 14 for PSEN2. EO-FAD mutations in these three genes leads to a common molecular phenotype, which represents an increase in the ratio of the $A\beta_{42}$:$A\beta_{40}$, being the $A\beta_{1-42}$ more willing to form aggregates and more toxic than the $A\beta_{1-40}$. One mutation in APP raises all species of Aβ and several others, resulting in amino acid substitution within the Aβ domain and leading to increased aggregation of Aβ (Table I). However, most EO-FAD mutations increase the $A\beta_{42}$:$A\beta_{40}$ ratio. The relative escalation in $A\beta_{42}$ promotes the aggregation of the peptide into oligomers and ultimately amyloid fibrils, which have neurotoxic properties [9].

Table I - Early-onset familial Alzheimer's disease (EO-FAD) genes and their pathogenic effects [9].

Gene	Protein	Chromosome	Mutations	Molecular phenotype
APP	Amyloid β (Aβ) protein precursor	21q21	24 (duplication)	Increased $A\beta_{42}/A\beta_{40}$ ratio Increased Aβ production Increased Aβ aggregation
PSEN 1	Presenilin 1	14q24	185	Increased $A\beta_{42}/A\beta_{40}$ ratio
PSEN 2	Presenilin 2	1q31	14	Increased $A\beta_{42}/A\beta_{40}$ ratio

Whereas EO-FAD is characterized by classic Mendelian inheritance usually in an autosomal-dominant manner, for LOAD, it is observed a genetically complex pattern of inheritance in which genetic risk factors work together with environmental factors and life exposure events to determine lifetime risk for AD.

The ApoE (Apolipoprotein E) polymorphic alleles, on chromosome 19q13, are the main genetic determinants of Alzheimer's disease risk in late-onset cases: individuals carrying the ε4 allele (ApoE ε4 allele) are at an increased risk of AD (20-25% of the Alzheimer's cases) compared with those carrying the ε3 allele (APOE ε3 allele), whereas the ε2 allele (ApoE ε2 allele) decreases the risk of the disease [9].

ApoE bind to several cell-surface receptors to deliver lipids, and also to hydrophobic amyloid-β (Aβ) peptide, which is thought to initiate toxic events that lead to synaptic dysfunction and neurodegeneration in Alzheimer's disease. ApoE isoforms differentially regulate Aβ aggregation and clearance in the brain, and have distinct functions in regulating brain lipid transport, glucose metabolism, neuronal signaling, neuroinflammation and mitochondrial function [9,10].

Other genes exhibit genome-wide significance for the association with LOAD: *CD33, GWA-14q31.2, ATXN1, CLU, PICALM, CR1, BIN1, ABCA7, MS4A6E/MS4A4E, CD2AP* and *EPHA1* [8,9].

According to amyloid (or Aβ hypothesis), neurodegenerative processes results from an imbalance between Aβ production and Aβ clearance, suggesting that other genes involved in these pathways (Table II) might also turn out to be risk factors [7].

Table II - Gene variants associated with Alzheimer's disease [7].

Gene	Main alteration	Presumed mechanism
Amyloid precursor protein (APP)	Mutation	Autosomal dominant, mostly early onset
Presenilin 1 (PSEN1)	Mutation	Autosomal dominant, mostly early onset
Presenelin 2 (PSEN2)	Mutation	Autosomal dominant, mostly early onset
Apolipoprotein E (ApoE)	Common variant	Familial and sporadic, late onset
Sortilin-related receptor, L(DLR class) A repeats-containing (SORL1)	Common variant	Familial and sporadic, late onset
Clusterin (CLU)	Common variant	Sporadic, late onset
Phosphatidylinositol binding clathrin assembly protein (PICALM)	Common variant	Sporadic, late onset
Complement componente (3b/4b) receptor 1 (CR1)	Common variant	Sporadic, late onset
Bridging integrator 1 (BIN1)	Common variant	Sporadic, late onset

- **Environmental and Other Factors**

The diet is one of the most important modifiable environmental factors associated with the risk of Alzheimer's disease. The study performed by Gu and colleagues [11] indicated that higher consumption of certain foods (salad dressing, nuts, fish, tomatoes, poultry, cruciferous vegetables, fruits, dark and green leafy vegetables) and lower of others (high-fat dairy, red meat, organ meat, and butter) may be associated with a decreased risk of developing AD via a more favorable profile of nutrients (i.e. lower ingestion of saturated fatty acids and higher ingestion of polyunsaturated fatty acids, vitamin E and folate). This is a characteristic of a composite dietary pattern such as the Mediterranean diet [7], that when combined with regular physical activity (compared with none or minimal), is associated with a significant reduction in the risk of AD. Exercise can enhance learning in both young and aged people, activate brain plasticity mechanisms, remodel neuronal circuitry in the brain, promote brain vascularization, and stimulate neurogenesis. It may

likewise increase neuronal survival and resistance to brain insults, increase levels of brain derived neurotrophic factor, mobilize gene expression profiles, that were predicted to benefit brain plasticity, and reduce levels of C-reactive protein (CRP) and interleukin-6 (IL-6), which are two inflammatory markers [7]. Also, individuals with intellectually enriched lifestyles, such as those with high educational and/or occupational attainment, have a reduced risk for AD pathology. Additionally, there is evidence for a protective role of leisure activities in dementia development, by improving lipid metabolism and mental stimulation [5,7].

There are other factors that modify the risk for Alzheimer's disease development such as cardiovascular diseases, sex or history of diabetes, hypertension, depression, smoking, obesity, dyslipidemia or traumatic head injury [5,7,12,13].

1.2.2 – Pathogenesis of AD

The major pathological features of Alzheimer's disease (AD) are the extracellular accumulation of β-amyloid (Aβ) peptide in the senile plaques (SP) (Figure 3A) and the intracellular accumulation of abnormally phosphorylated tau protein (Figure 4B) as neurofibrillary tangles (NFT) (Figure 3C) [2].

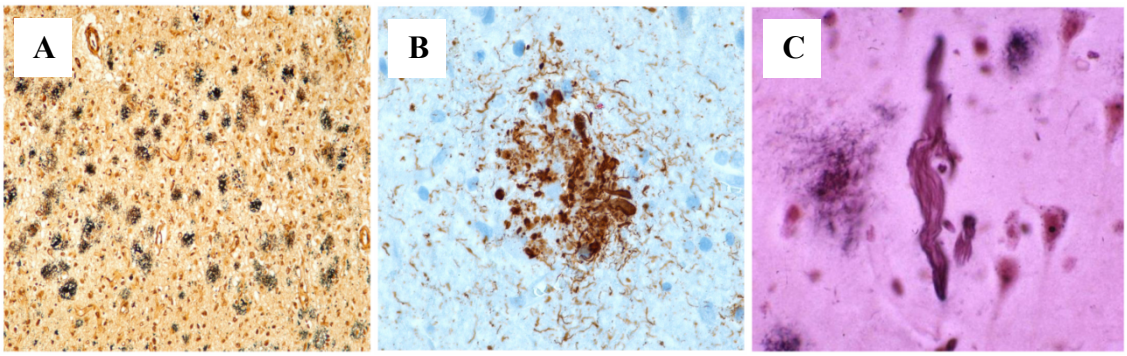

Figure 3 – Pathological features of Alzheimer's disease (AD). (A) Senile plaques (SP) are demonstrated in Bielshowsky silver impregnation; **(B)** Immunohistochemical stain for phosphorylated tau (monoclonal antibody AT8 which recognizes phosphorylation at residues 202 and 205) shows numerous distended neurites in a classic neuritic plaque (a senile plaque subtype) and **(C)** Neurofibrillary tangles (NFT), as seen on the Bielshowsky silver stain, were initially characterized by Alzheimer (adapted from [5]).

- **APP and Aβ genesis**

Amyloid Precursor Protein (APP) is one of the three members of a small gene family, which includes the Amyloid Precursor Like Protein (APLP) 1 and APLP2 (human), but only APP contains sequence encoding the Aβ domain [14]. It is a ubiquitously expressed type I membrane glycoprotein that is encoded by a single gene on the chromosome 21q21 and multiple isoforms exist resulting from alternative splicing. The predominant transcripts are APP_{695}, APP_{751} and APP_{770}. APP is expressed in many cells and tissue types including endothelia, glia and neurons. Whereas APP_{695} is the predominant isoform in neuronal cells, APP_{751} and APP_{770} are predominantly expressed in peripheral tissues.

From the proteolytic processing of the APP, derives the Aβ peptide. APP can be cleaved by two different pathways (Figure 4). In **amyloidogenic pathway**, APP is processed by β-secretase that generates the soluble fragment sAPPβ and a membrane associated C terminal fragment consisting of 99 amino acids (C99). C99 is a substrate for γ-secretase, leading to the release of the APP intracellular domain (AICD) and generating Aβ peptide, which aggregates and fibrillates to form amyloid plaques in the brain [15]. Alternatively, in the **non-amyloidogenic pathway** APP is sequentially cleaved by α-secretase and γ-secretase. α-secretase originates the soluble APP-fragment sAPPα and a membrane-associated C-terminal fragment consisting of 83 amino acids (C83). APP C83 is further cleaved by γ-secretase to release a P3 peptide and the AICD, both of which are rapidly degraded.

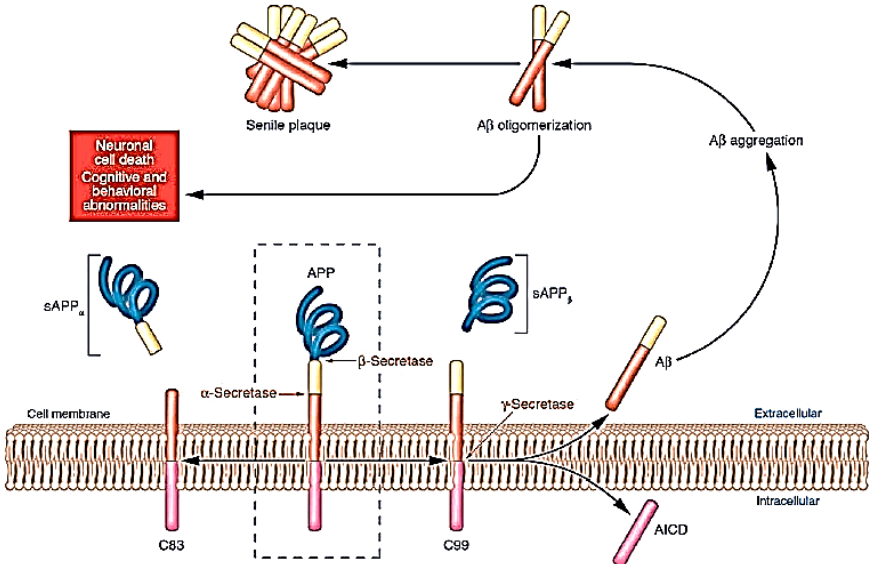

Figure 4 – APP processing and Aβ accumulation. Mature APP (*center, inside dashed box*) is metabolized by two competing pathways, the α-secretase or non-amyloidogenic pathway that generates sAPPα and C83 (also known as CTFα; *left*) and the β-secretase or amyloidogenic pathway that generates sAPPβ and C99 (*right*) (taken from [16]).

Aβ overproduction and aggregation into SP, appear central to the clinical manifestation of Alzheimer's disease, both in sporadic and familial early-onset cases [6]. Aggregates of the Aβ peptide accumulate in the plaques and vessel walls [2] and of the several Aβ species found in AD, $A\beta_{42}$ rather than $A\beta_{40}$, represents the predominant form of the peptide deposited in SP, whereas $A\beta_{40}$ is more likely involved in vascular amyloid deposition [6].

- **Tau and NFTs formation**

Neurofibrillary tangles, the other major well-recognized pathological hallmark of Alzheimer's disease, accumulates early in AD brains. Caused by abnormal hyperphosphorylation of tau by the putative kinases, glycogen synthase kinase-3β (GSK-3β) and cyclindependent kinase 5 (cdk5), NFT accumulate intracellularly in the neuronal cytoplasm [15]. Tau, is the most abundant 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. On the other hand, hyperphosphorylated tau (p-tau) displays a strong tendency to abnormally aggregate, leading initially to paired helical filaments formation and then to larger NFT. Microtubular and cytoskeletal instability due to p-tau and NFT formation, leads to impaired axonal transport to presynaptic and postsynaptic terminals, causing energy and neuronal nutritional depletion at these sites. This in turn results in neuronal degeneration [6].

The most influential theory to explain the pathogenesis of AD has been the "Amyloid Cascade Hypothesis" (ACH) first formulated in 1992. The ACH proposes that the deposition of Aβ is the initial pathological event in AD, which leads to the formation of senile plaque, then to neurofibrillary tangles, death of neurons and ultimately dementia. Nevertheless, there are observations that are difficult to reconcile with this hypothesis, thus a modification of the original ACH was proposed to better explain the pathogenesis of the AD [17,18]. In this modified hypothesis (Figure 5), the essential trigger to the development of AD is brain ageing and the associated risk factors such as head trauma, vascular disease, and systemic disease that are referred to as the "allostatic load". These factors exacerbate processes leading to cell death. Genetic factors, rather than initiating disease, indirectly influence the formation and composition of peptides, formed when neurons degenerate. As neurons degenerate, various proteins are upregulated leading to the formation of

extracellular Aβ deposits and intracellular tau phosphorylation, latter resulting in the NFT development. These reaction products may be toxic and initiate a further phase of secondary degeneration that accelerates the neuronal loss leading to dementia [17]. Significant neuronal loss occurs in the cholinergic nuclei of the basal forebrain, which projects widely to cortical areas and accounts for the observed cholinergic neurotransmission deficits that contribute, in part, to memory decline [6].

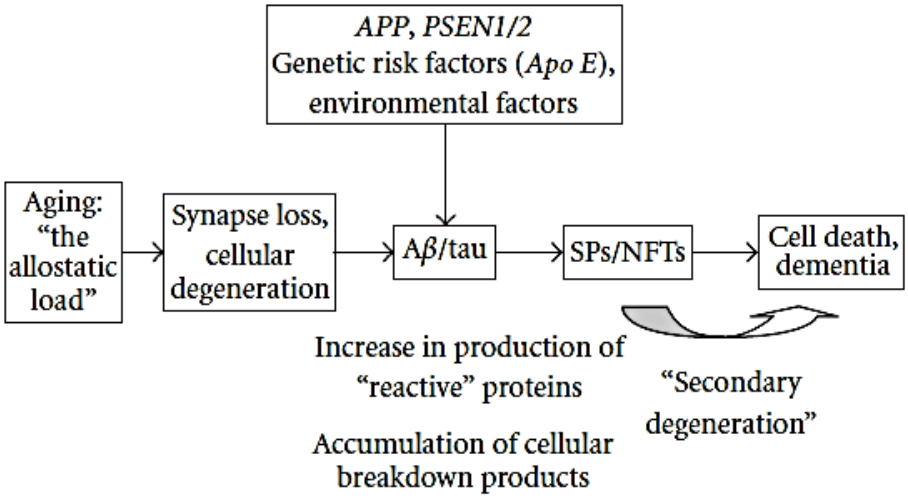

Figure 5 - A modification of the "Amyloid Cascade Hypothesis" (ACH) (taken from [17]).

1.2.3 – Diagnosis of AD

In 1984 representatives from the *National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association* (NINCDS-ADRDA) developed a uniform set of criteria to enable clinicians and researchers to maintain consistency in the Alzheimer's disease diagnosis. These included aspects of medical history, clinical examination, neuropsychological testing, and laboratory assessments. However, these NINCDS-ADRDA criteria were recently updated due to the advances in neuropsychological assessment, brain imaging and the neuropathological, biochemical and genetic understanding of the disease. The growing use of brain imaging and cerebrospinal fluid (CSF) biomarkers, aid in the specificity and sensitivity of the diagnosis and thus are considered in the updated diagnostic criteria, especially when used for clinical research [7].

Alzheimer's disease diagnosis requires clinical evidence of memory loss and impairment of at least one other cognitive domain, with evidence of disturbance in social or occupational function [5]. The global indices of cognitive performance and global functional status could be obtained using both the Mini-Mental State Examination (MMSE) and the Clinical Dementia Rating (CDR) [19].

The **neuroimaging techniques** such as Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) may potentiate the diagnosis of AD [20]. Neuropathological studies have documented an abundance of NFT and significant neuronal loss in the hippocampus and entorhinal cortex of AD patients. Therefore, these have been the areas frequently targeted by imaging techniques [21,22].

MRI is useful for neuroimaging examination for Alzheimer's disease because it allows an accurate measurement of the 3-dimensional (3D) volume of the brain structures, especially the size of the hippocampus and related regions (Figure 6A) [23]. PET has been employed in many AD studies to examine the regional cerebral metabolic rate for glucose (rCMRGLc) using 18F-2-deoxy-2-fluoro-D-glucose (FDG) as a marker. Reduction of glucose metabolism, observed on PET in the bilateral temporal parietal regions and in the posterior cingulate (Figure 6B), is the most commonly described diagnostic criteria for AD. PET imaging using 11C-labeled PIB {2-[4'-(methylamino) phenyl]-6-hydrobenzothiazole} ligand has also been one of the major diagnostic tools more recently developed for AD. PIB binds with high affinity and high specificity to fibrillar Aβ in neuritic plaques and cerebral amyloid angiopathy (Figure 6C). In AD patients, PIB retention is increased in the frontal, parietal, temporal and occipital cortices, and in the striatum [21].

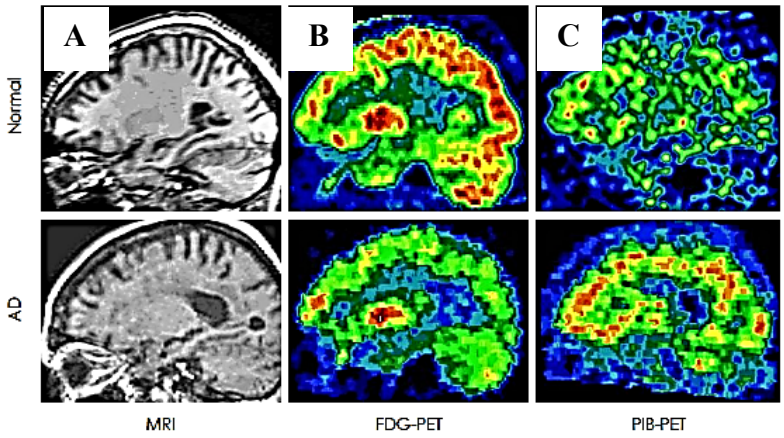

Figure 6 – Neuroimaging techniques in two representative cases. (A) MRI; **(B)** FDG-PET; and **(C)** PIB-PET of a normal control (*top row*) and an Alzheimer's disease patient (*bottom row*). MRI: Magnetic Resonance Image; PET: Positron Emission Tomography (adapted from [24]).

Since deposition of β-amyloid (Aβ) peptide is known to precede clinical signs of dementia, PIB-PET may facilitate the early detection of amyloid during preclinical AD. The cognitively normal elderly people demonstrate substantial PIB retention in the cortex by their mid-70s, and this PIB retention is similar in extent to that of patients with mild-to-moderate AD. Brain amyloid can be assessed based on reductions in the CSF $A\beta_{42}$ levels and increased PIB retention while an elevated CSF tau is thought to be a biomarker of tau-mediated neuronal injury and neurodegeneration. Decreased FDG uptake on PET in the temporoparietal area is a biomarker of AD-related synaptic dysfunction, and brain atrophy seen on structural MRI involving the medial temporal lobe is a biomarker of AD-related neurodegeneration. Structural MRI is the last biomarker to become abnormal [21,25].

Different **biomarkers** for Alzheimer's disease may potentially be complementary in diagnosis and prognosis of AD [20]. Nowadays a definitive diagnosis can only be made on histological examination of the brain at autopsy [5]. Thus the identification of individuals with preclinical AD before the development of cognitive deficits and concomitant neuronal loss is fundamental to a timely therapeutic intervention [21].

There are two basic types of biomarkers in Alzheimer's disease: (a) trait biomarkers that define a risk state for AD but do not necessarily indicate that the disease is present and (b) state biomarkers that are indications of pathological changes of AD [26].

Genetic biomarkers, such as autosomal dominant AD-causing mutations and the Apolipoprotein E ε4 (ApoE ε4), and other risk polymorphisms are examples of trait biomarkers. State biomarkers indicate the presence and/or the severity of AD and they can be used for diagnosis, staging, or outcomes assessment. The principal state biomarkers include atrophy on MRI; reduced metabolism on FDG-PET; and more recently, amyloid deposition on amyloid imaging and changes in CSF levels of total tau (t-tau), hyperphosphorylated tau (p-tau), or $A\beta_{1–42}$ (Figure 7) [26].

The cortical axonal degeneration that occurs in AD brains, results in increased CSF levels of t-tau, which predicts a rapid cognitive decline. Regarding the CSF levels of p-tau protein, they are the most specific finding suggesting an ongoing AD process in the brain and are also increased [27]. CSF $A\beta_{1-42}$ levels are commonly decreased in patients with Alzheimer's disease, possibly because it is the main component of AD plaques, which may function as sinks or traps, thus decreasing the amount of $A\beta_{1-42}$ cleared from the brain to

the cerebrospinal fluid (Table III) [21].

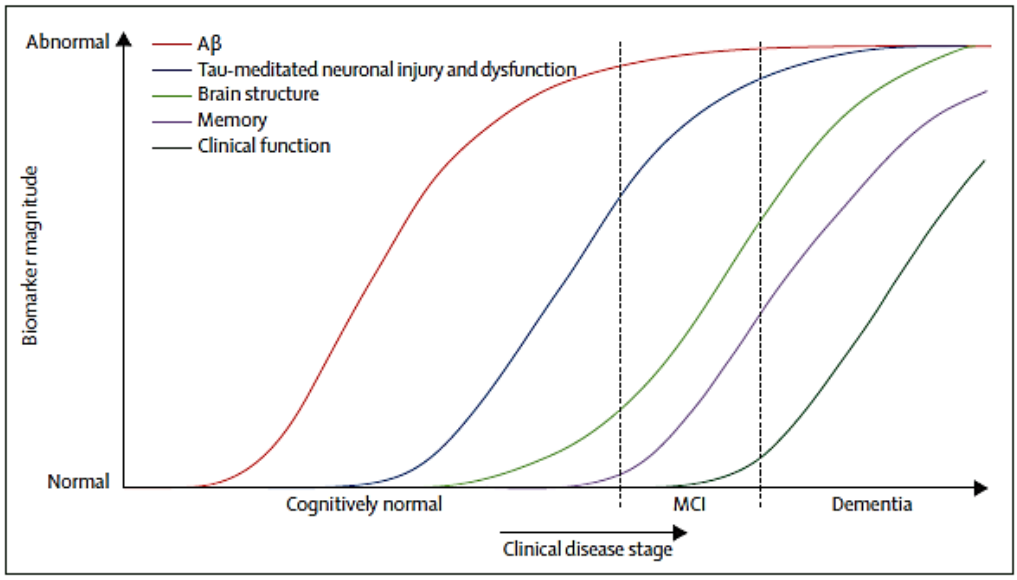

Figure 7 - Dynamic biomarkers of the Alzheimer's pathological cascade. β-amyloid peptide (Aβ) is identified by cerebrospinal fluid (CSF) $A\beta_{42}$ or PET amyloid imaging. Tau-mediated neuronal injury and dysfunction is identified by CSF tau or fluorodeoxyglucose-PET. Brain structure is measured by use of structural MRI. PET: Positron Emission Tomography; MRI: Magnetic Resonance Imaging; MCI: Mild Cognitive Impairment (taken from [28]).

The routine diagnosis of AD based on cerebrospinal fluid has several drawbacks: lumbar puncture and collection of CSF is an invasive treatment with potential side effects, screening of patients is often difficult and follow-up analysis of the same patient over several years is problematic. Thus, recent researches have been focusing in the search for biomarkers in other body fluids, such as blood, in order to diagnose AD. However, it is still unknown how the concentration of analytes in the blood, more precisely in plasma, directly correlates with pathological changes in the AD brain [29].

Table III - Fluid biomarkers of Alzheimer's disease (AD). β-amyloid (Aβ) 42, total tau (t-tau) and hyperphosphorylated tau (p-tau) levels in plasma and cerebrospinal fluid (CSF). *Up arrow* = Increase; and *Down arrow* = Decrease.

ALZHEIMER'S DISEASE (AD)			
	Plasma	**CSF**	**References**
$A\beta_{42}$	↑	↓	[29], [21], [30], [25], [31]
t-tau	Less elevated	↑	[29], [31], [32], [33], [34], [35]
p-tau	Less elevated	↑	

The pathogenic process of AD probably starts decades before clinical onset of the disease. During this preclinical period, there is a gradual neuronal loss and the first symptoms, most often impaired episodic memory, appear at a certain threshold. This clinical phase is often designated as mild cognitive impairment (MCI) [33]. There are two terminological systems to identify AD stages (Table IV). The *International Work Group* identifies at-risk cognitively normal populations, prodromal AD, and AD dementia. While the approach of the *National Institute on Aging/ Alzheimer's Association* describes preclinical AD, MCI of the AD type (with intermediate or high likelihood of AD), and AD dementia (with intermediate or high likelihood of AD) [26,32].

Table IV – Classification of the Alzheimer's disease stages. Aβ: amyloid-β; AD: Alzheimer's disease; ADLs: activities of daily living; MCI: mild cognitive impairment (adapted from [26]).

Features	**International Work Group criteria**	**National Institute on Aging/Alzheimer´s Association criteria**
Normal cognition; intact ADLs; biomarker evidence of AD	At-risk state with AD pathology	Preclinical AD
Abnormal cognition; normal ADLs; biomarker evidence of AD	Prodromal AD	MCI due to AD; intermediate likelihood: one biomarker present (Aβ or neuronal injury); high likelihood: two biomarkers present (Aβ plus neuronal injury)
Abnormal cognition; abnormal ADLs; biomarker evidence of AD	AD dementia	AD dementia; intermediate likelihood: one biomarker present (Aβ or neuronal injury); high likelihood: two biomarkers present (Aβ plus neuronal injury)

The development of validated biomarkers for AD is essential to improve its diagnosis and accelerate the development of new therapies. Biochemical and neuroimaging markers could facilitate diagnosis, predict AD progression from a pre-AD state of MCI, and be used to monitor efficacies of disease-modifying therapies [19].

Other biomarkers associated to relevant processes for AD, such as those involved in inflammation, are also being tested due to the possible link with the disease pathogenesis.

1.3 – Inflammation in AD

Inflammation is a body response to eliminate the initial cause of cell injury as well as necrotic cells and tissues resulting from original insult. The cells responsible for the inflammatory reaction are microglia, astrocytes, and neurons that when activated produce high levels of inflammatory mediators such as pro-inflammatory cytokines and chemokines. Prostaglandins, leukotrienes, thromboxanes, coagulation factors, free radicals as reactive ROS and nitric oxide (NO), complement factors, proteases and protease inhibitors, and C-reactive protein (CRP) are also produced by them [6,12,36]. Brain inflammation is a pathological hallmark of Alzheimer's disease. Robust evidences of disturbances in inflammation and immune pathways in the disease is strongly associated with increased levels of acute-phase proteins and pro-inflammatory cytokines in blood, CSF and post-mortem brain tissue of AD patients [37].

An acute insult is typically short-lived and unlikely to affect the long-term neuronal survival. Moderately active microglia is thought to perform beneficial functions, such as scavenging neurotoxins, removing dying cells and cellular debris, and secreting trophic factors that promote neuronal survival [38].

In contrast, chronic neuroinflammation is a long-standing and often self-perpetuating neuroinflammatory response that persists long after an initial injury or insult. Chronic neuroinflammation includes long-standing activation of microglia and subsequent sustained release of inflammatory mediators. This works to perpetuate the inflammatory cycle, activating additional microglia, promoting their proliferation, and resulting in further release of inflammatory factors, which in turn leads to elevated levels of several cytokines [38].

Signs of chronic inflammation occur in pathologically susceptible regions in AD brains as a response to Aβ peptide deposition and NFT formation. One hypothesis is that both Aβ plaques and tangles stimulate a chronic inflammatory reaction. In turn, inflammatory mediators, enhance APP production and the amyloidogenic processing of APP to induce increased $A\beta_{42}$ peptide production. These circumstances also inhibit the generation of a soluble APP fragment that has a neuroprotective effect. On the contrary, Aβ induces the expression of pro-inflammatory cytokines, such as interleukins (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in glial cells, leading to a vicious cycle [12,38]. Therefore, the neuroinflammatory process triggered by $A\beta_{42}$, plays a central role in the

neurodegenerative process. This inflammatory process leads to both synaptic and neuronal damage as well as further inflammatory cell activation [6,29]. The activation of microglia results in a phagocytic phenotype and the release of inflammatory mediators that promote the inflammatory state [38].

Neurodegeneration and neuroinflammation can result in changes of the central nervous system (CNS) proteins (for example, Aβ peptide) or inflammatory mediators across the Blood Brain Barrier (BBB). These CNS-derived proteins and mediators may induce systemic immune reactions and/or recruit lymphocytic cells into the CNS. Other than CNS resident cells, blood-derived cells can also be blamed for inflammatory response and seem to accumulate in the AD brain due to the expression of chemokine receptors [12] (Figure 8).

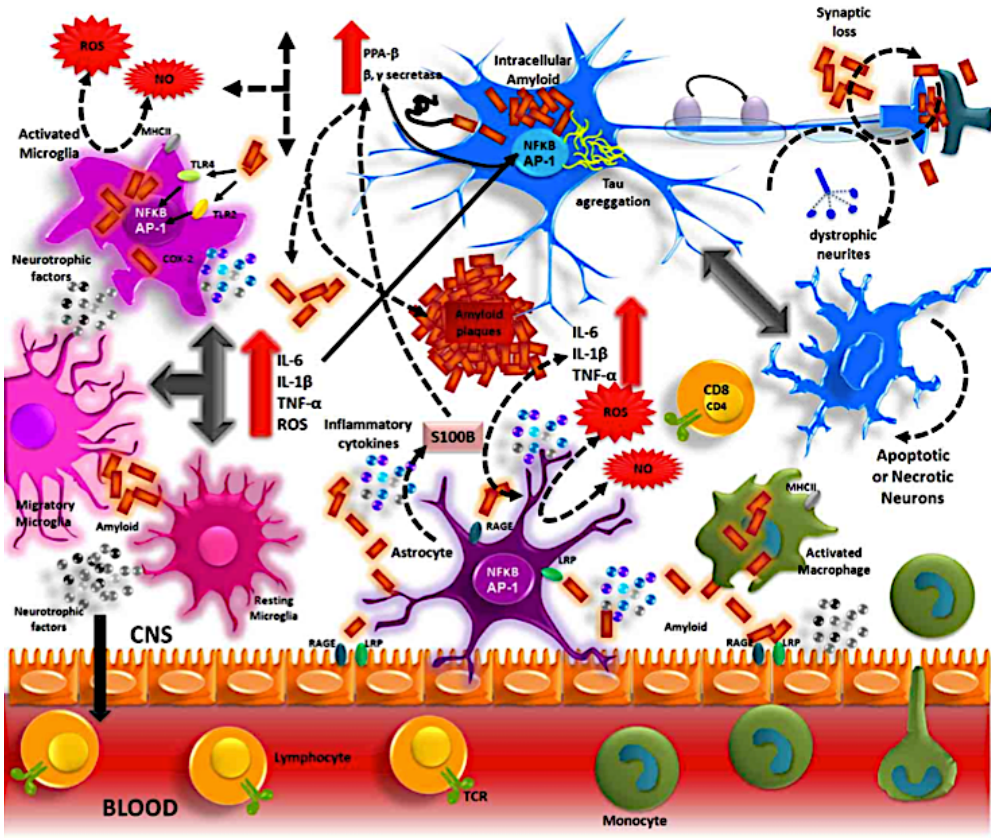

Figure 8 – Neuroinflammation process in AD. During Alzheimer's disease, Aβ molecules accumulate and forms mature Aβ plaques (SP). In response to the deposition of Aβ and the release of chemoattractants from damaged neurons, activated astrocytes, microglia, and macrophages release pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. These inflammatory compounds amplify the pro-inflammatory response, producing neurotoxic effects. Adhesion molecules and chemokines are also produced, being responsible for the recruitment of peripheral immune cells (taken from [39]).

- **Interleukins in AD**

Interleukins are an important class of inflammatory cytokines in many organs and tissues. They direct the inflammatory response within the CNS by activating several intracellular signalling pathways within multiple CNS cell types, as well as coordinating signalling between neurons and astrocytes. Several cytokines thought to be important in AD pathogenesis have been found upregulated in vulnerable areas in patient's brains [6,40].

The most potent cytokines are interleukin-1α (IL-1α), IL-1, IL-6 and TNF-α [36]. Swardfager and collaborators [41] performed a meta-analysis to study the cytokines present in AD. Obtained data revealed higher peripheral concentrations of IL-6, TNF-α, IL-1β, transforming growth factor-β (TGF-β), IL-12 and IL-18 and higher TGF-β concentrations in the CSF.

Further, it was also shown that interleukin-1 receptor antagonist (IL-1ra), IL-6 and IL-10 were significantly associated with ventricular volume in AD [42]. Of note abnormal cytokine levels can also impact on APP, for instance, inflammatory cytokines such as IL-1β, IL-6, TNFα, or interferon (IFN) gamma can augment APP expression and Aβ formation [8].

There is a feedback signalling loop between Aβ and IL-1β: Aβ can induce the production of IL-1β, whose presence greatly increases the secretion of the cytokine IL-6 and chemokine IL-8 in response to Aβ by astrocytes. On the other hand, both IL-1α and IL-1β upregulate the expression of APP, potentially enhancing the production of Aβ. However, IL-1β may also activate the non-amyloidogenic pathway [38].

IL-18 likewise, significantly elevated in brain specimens from AD patients, can increase the amyloid plaque formation by inducing the expression of PSEN1 and the β-site APP-cleaving enzyme 1 (BACE1), involved on APP amyloidogenic processing. Additionally, IL-18 can increase levels of Cdk5/p35 and GSK-3 kinases, leading to tau hyperphosphorylation [43].

- **Chemokines in AD**

Chemokines are a family of conserved chemotactic cytokines able to induce the migration of several leukocyte subpopulations into damaged tissues. Chemokines are classified into four families: CC, CXC, CX3C and C [44], and in the adult brain, microglia and astrocytes are believed to be the main source of chemokine production. The CNS has been shown to

produce chemokines in response to several inflammatory and disease conditions, including Alzheimer's disease [45].

Exposure of microglial cells to Aβ causes their activation and leads to the production not only of pro-inflammatory cytokines and but also of chemokines, such as macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1) and IL-8. Astrocytes, the most common cells in the brain, can also be activated by Aβ peptides to synthetize various pro-inflammatory molecules similar to those produced by microglia [44].

In particular, MCP-1 (also named CCL2) is produced by microglial cells and stimulates astrocytes, which together participate in the degradation of Aβ peptide. Significantly increased MCP-1 levels were found in MCI and mild AD, but not in severe AD patients when compared with controls. More, evidences indicate that plasma MCP-1 levels could serve as a biomarker to monitor inflammatory process of AD [46]. Porcellini and colleagues [47] proposed that, decreased levels of peripheral MCP-1 along with increased CSF concentration of this chemokine in AD, may suggest impaired MCP-1 turnover between these two compartments.

Besides MCP-1, reactive astrocytes have been shown to secrete other pro-inflammatory mediators such as RANTES (regulated on activation, normal T-cell expressed and secreted) or CCL5 in response to stimulation with $A\beta_{42}$. In turn, both chemokines attract microglial cells, whose further expression of pro-inflammatory products, leads to additional neuronal damage [44].

Further, both CC chemokines, are found on endothelial cells, glia and neurons throughout the brain, suggesting important functions for this inflammatory superfamily in the CNS. RANTES could be correlated with Th1 (helper T cell) responses, since it is thought to promote leukocyte infiltration in sites of inflammation and activate T cells. MCP-1 however, promotes attraction and activation of monocytes, activates T cells, NK cells (Natural Killer cells), and basophils, playing a key role in trans-endothelial migration of inflammatory cells [43].

IL-8, a chemokine produced by macrophage and other cell types in response to pro-inflammatory mediators such as Aβ, could be important for the recruitment of activated microglia into sites of damaged brain. CXCR2, an IL-8 receptor, has been localized in dystrophic neurites (defined as thickened or irregular neuronal processes), suggesting that

IL-8 mediates glial interactions with neurons thereby contributing to neuronal damage. IL-8 was significantly increased in the CSF of AD compared to control individuals [48], whereas plasma levels in late-onset AD did not differ significantly from controls. In contrast, an independent and negative relationship between plasma IL-8 levels and MCI and AD patients was showed by Kim *et al* [46]. Mariani *et al* [44], showed that CSF levels of both MCP-1 and IL-8 were higher in patients with MCI and AD.

In a study performed by Ray *et al* [49], a group of plasma proteins, including many cytokines and chemokines such as IL-8 and RANTES were proposed to differentiate AD patients from controls. More recently Soares and colleagues [50] showed that some of those proteins could be detected by bead-based multiplex technology, suggesting a plasma-based AD signature that may be a useful screening tool.

Table V summarizes the data obtained for some cytokines in AD patients.

Table V – Examples of cytokines levels in Alzheimer's disease (AD). Interleukins and chemokines levels in plasma, cerebrospinal fluid (CSF) and AD brains. IL: interleukin; TNF: tumor necrosis factor; IFN: interferon; MCP: monocyte chemotactic protein; RANTES: regulated on activation, normal T cell expressed and secreted. *Up arrow* = Increase; *Down arrow* = Decrease; *Equalaty symbol* = Unaltered; and *Dashed* = No information.

	Cytokines	Plasma/ Serum	CSF	AD brains	References
Interleukins	IL-1β	=↑	↑	↑	[51], [52], [53], [54], [42]
	IL-18	=↑	↑	=↑	[55], [56], [57], [58]
	IL-6	=↑	↓↑	↑	[51], [52], [53], [59], [60], [61]
	TNF-α	↓↑	=	↑	[62], [63], [64]
	IFN-γ	=	=	---	[65], [61]
Chemokines	IL-8	=↓	↑	↑	[42], [66], [46]
	MCP-1 (CCL2)	=↓	=↑	↑	[53], [42], [46], [67], [68], [63]
	RANTES (CCL5)	↑	---	↑	[69], [70], [43]

The presence of inflammatory molecules is likely to be a very early event in AD pathogenesis, preceding the disease clinical onset. However, it has been proposed that their chronic production in advanced Alzheimer's disorder could be harmful and contributes to neuronal death [44]. This may be related with abnormal APP processing and tau phosphorylation in AD (Table VI).

MCP-1 overexpression was shown to induce microgliosis and enhance Aβ deposits by reducing their clearance by increasing Apolipoprotein E (ApoE) expression levels. As mentioned, Aβ aggregation promotes diffuse plaque formation and is associated with neuronal death and consequently cognitive impairments [71,72]. By contrast, MCP-1 also enhances Aβ uptake by microglia, which leads to an increase in oligomerized Aβ species in both intracellular and extracellular fractions. Microglia-induced Aβ internalization occurs through a nonsaturable, fluid phase macropinocytic mechanism, that is dependent on actin and tubulin dynamics of the cellular cytoskeleton. Therefore, this suggests that MCP-1 may prominently activate cytoskeletal dynamics as a part of its chemotactic signalling and thereby affect the Aβ uptake [73].

Table VI – Examples of cytokines effects on Aβ peptide, APP and tau. IL: interleukin; TNF: tumor necrosis factor; IFN: interferon; MCP: monocyte chemotactic protein; RANTES: regulated on activation, normal T cell expressed and secreted; sAPP: soluble APP fragment; AICD: APP intracellular domain; BACE1: β-site APP-cleaving enzyme 1; p-tau: hyperphosphorylated tau. *Up arrow* = Increase; *Down arrow* = Decrease; and *Dashed* = No information.

	Cytokines	Aβ	APP processing	APP & tau Phosphorylation	References
Interleukins	IL-1β	↑ Aβ	↑ sAPPα; ↑ γ-secretase activity & AICD	↑ APP mRNA; ↓ full-length APP; ↑ tau mRNA and p-tau	[74], [75], [76], [77], [78], [79]
	IL-18	↑ $A\beta_{1-40}$	↑ sAPPβ and BACE 1	↑ p-APP and p-tau	[80], [81]
	IL-6	---	---	↑ APP mRNA ↑ p-tau	[82], [83]
	TNF-α	↑ Aβ deposition	↑ sAPPβ; ↑ γ-secretase activity & AICD	↑ APP mRNA ↑ p-tau	[79], [84], [85], [86]
	Interferon-γ	↑ Aβ	↑ γ-secretase activity & AICD	↓ p-tau	[79], [86], [87]

Chemokines	IL-8	---	---	---	[88], [89]
	MCP-1 (CCL2)	↑ Aβ deposition ↑ Aβ uptake & oligomerization	---	---	
	RANTES (CCL5)	---	---	---	

Progressive Aβ deposition in the brain, as a cause of inflammation and abnormal APP processing, can induce disruption of mitochondrial membrane, resulting in ROS production, release of cytochrome c from mitochondria and activation of caspase-dependent apoptotic pathways and consequently neurodegeneration [90].

Cytokines per se can trigger apoptotic processes. As an example, a study performed by Thirumangalakudi *et al* [91], showed that IL-8 is able to induce the expression of the pro-apoptotic protein Bim leading to neuronal death. Bim is a part of the pro-apoptotic Bcl-2 family of proteins that regulate the apoptosis by controlling the mitochondrial permeability [92,93].

In summary, inflammatory mediators such as pro-inflammatory cytokines and chemokines are present in brain inflammation and represent a pathological hallmark of the Alzheimer's disease. However, as observed in Table VI, little is known about the role of chemokines in AD pathogenesis, namely if these chemokines can affect APP processing and tau phosphorylation, as well as their impact on apoptosis.

Hence, additional studies are needed to improve our knowledge about this field in order to discover new possible inflammatory biomarkers candidates for AD diagnosis or novel targets for therapeutic intervention.

2 – Objectives

2 – Objectives

Alzheimer's disease is a complex neurodegenerative disorder characterized by the presence of SP and NFT, synaptic loss and consequently neurodegeneration. The Aβ peptide is the major constituent of SP and gradual Aβ production and aggregation leads to the activation of inflammatory responses, which plays a crucial role in AD progression.

Inflammatory mediators such as pro-inflammatory cytokines and chemokines are highly produced by microglia, astrocytes and neurons, during the inflammatory process associated to AD. The initial inflammatory response is beneficial and needed to prevent the neurotoxicity caused by the amyloid fragments and to limit the disease progression. However, the sustained release of inflammatory mediators works to perpetuate the inflammatory cycle, activating additional microglia, promoting their proliferation, and resulting in further release of inflammatory factors, leading to elevated levels of several cytokines. In an advanced AD phase, due to the persistent activation of microglia, chronic neuroinflammation occurs, adding in the neurotoxic effects induced by Aβ and leading to neuronal death. In particular, the role of chemokines in AD pathogenesis is unclear. Hence the aim of this project was to evaluate how IL-8 and MCP-1 impacts on apoptosis and the two main AD-related proteins, APP and tau.

Therefore, the specific aims included:

i. Evaluate the IL-8 and MCP-1 cytotoxicity;
ii. Evaluate the effects of the IL-8 and MCP-1 on the Amyloid Precursor Protein (APP) processing;
iii. Determine the impact of the IL-8 and MCP-1 on tau and other cytoskeleton-associated proteins.

3 – Materials and Methods

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3.1 – Cell culture

All procedures involving cell culture and manipulation were performed using a class II air flow cabinet. SH-SY5Y human neuroblastoma cells are originally derived from the cell line SK-N-SH, established from a bone marrow biopsy of a neuroblastoma patient. SH-SY5Y cells cryopreserved in liquid nitrogen (-196ºC) were quickly thawed using a water bath at 37ºC, and then collected into a conical tube. The culture medium was added dropwise (to avoid cell lysis) and re-suspended to dilute the dimethyl sulfoxide (DMSO) present in the cell-freezing medium. The cells were centrifuged at 1000 rpm, for 3 min and the supernatant removed. The pellet was re-suspended in minimal essential medium (MEM):F12 (1:1) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (200 mM stock solution), sodium bicarbonate, sodium pyruvato and 1% of antibiotic/antimycotic mix. The cells were then seeded in a culture plate and medium replaced in the next day to remove dead cells. Cells were maintained in an incubator, at 37ºC with 5% CO_2/95% air and 95% humidity and passaged when reached approximately 70-80% of confluence until submitted to experimental procedure.

3.1.1 – Differentiation of the SH-SY5Y cell line

An optimization of the SH-SY5Y differentiation protocol was carried on before experimental procedures with differentiated cells. For differentiation of SH-SY5Y cell line, cells were detached with trypsin from the culture plate, count with a hemocytometer and seeded at a density of $5x10^5$ cells/well (6-well plate) or $0.5x10^5$ cells/cm^2. Cells were maintained in an incubator, at 37ºC with 5% CO_2/95% air and 95% humidity, during two days in 1% FBS culture medium supplemented with 10 μM of retinoic acid (RA – 20 mM stock solution).

3.1.2 –Chemokine treatment of SH-SY5Y

To test the effects of the chemokines in SH-SY5Y neuroblastoma cells, the lyophilized interleukin-8 (IL-8) and Monocyte Chemoattractant Protein-1 (MCP-1) (Both from BD Biosciences) were reconstituted in distilled water, aliquoted and stored at - 80ºC.

For the experimental procedure, IL-8 and MCP-1 (both with 200 ng/µL stock solution) were successively diluted in serum free culture medium, both at the final concentrations of 10, 50, 100, 150 ng/mL (Figure 9) and added to the culture cells, for different time periods (IL-8: 24 and 48 hours; MCP-1: 6 and 24 hours).

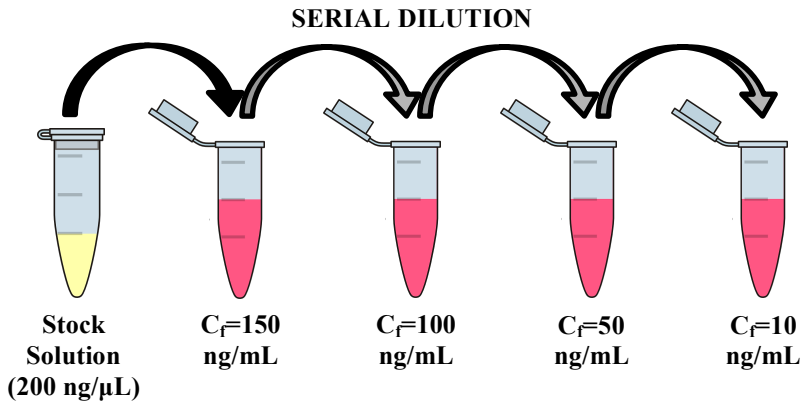

Figure 9 – Representative scheme of the serial dilution. Cf: Final concentration.

3.2 – Cell viability

Cell viability was determined using the MTT assay. MTT is a colorimetric assay developed by Mossman, that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble colored (dark purple) formazan product. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of cell viability.

SH-SY5Y cells were seeded at a density of $1x10^5$ cell/well in 24-well plates, differentiated and incubated in serum free culture medium with crescent concentrations of IL-8 and MCP-1 (10, 50, 100 and 150 ng/ml), during 24 and 48 hours. After this time, the culture medium was removed and replaced by 250 µL of serum-free culture medium with

25 µL of MTT (C_f = 0.5 mg/ml). Cells were then incubated at 37ºC for 4 hours. A negative control was included (25 µL of the MTT added to 250 µL of the medium alone). After incubation, medium was removed and the formed precipitates (dark purple) dissolved with 250 µL of 99.9% DMSO by vigorously mixing with a pipette. The solutions from each well were transferred to a 96-well plate and the absorbance read at 570 nm in the Infinite M200 (Tecan).

3.3 – Samples collection and Immunodetection

After the appropriated treatments with IL-8 and MCP-1, culture media was collected and centrifuged at 300g, 4ºC, during 5 min, to avoid cellular contamination, and then diluted with SDS 10%, to a final concentration of SDS 1%.

Cells were collected with RIPA buffer composed by Sodium Fluoride (NaF), Sodium Orthovanadate (NaO) and a protease inhibitor cocktail, using a cell scraper. After, cell lysates were sonicated twice, during 5 seconds, to obtain homogenous samples. All samples were stored at -20ºC before their preparation for SDS-PAGE. Culture media was used for sAPP detection and cell lysates for APP and tau detection.

3.3.1 – Determination of the protein concentration

For cell lysate protein quantitation the bicinchoninic acid (BCA) protein assay (Pierce), based on the use of the BCA, for the colorimetric detection and quantitation of the total protein, was used. This method combines the capability of the 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 give origin to a purple-colored reaction product, which exhibit a strong absorbance at 562 nm.

The quantitative analyses were performed using 5 µL of the collected cell lysates and a standard curve, prepared was described in Table VII. The samples and standards were incubated with 200 µL of working reagent (mixture of reagent A with reagent B in the proportion of 50:1) and incubated at 37ºC during 30 min. After some minutes (in order to cool down the samples), the absorbance was measured at 562 nm in the Infinite M200

(Tecan). The I-control™ software was used for data acquisition. Protein content was normalized and samples submitted to SDS-PAGE.

Table VII – Standards used in the BCA protein assay method. Bovine Serum Albumin (BSA) solution at 2 mg/ml. SDS, Sodium dodecyl sulfate.

Standard	BSA (µl)	1% SDS (µL)	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 – SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared to separate proteins according to their molecular weight and negative charge. The principle of the SDS-PAGE relies on the capacity of the 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 their migration toward the positively charged anode. SDS is also used in combination with a reducing agent (mercaptoethanol) and heated to dissociate proteins before loading on the gel. SDS also breaks up aggregates and non-covalently bound multimers.

Gels comprised two phases: in the top, the non-restrictive large pore called stacking gel and in the bottom, the resolving gel with a linear progression of acrylamide concentrations (gradient gel 5 to 20%), resulting in a wider separation range. The resolving

gel (Table VIII) was prepared and inserted into the electrophoresis system. After 45 min of polymerization at room temperature (RT), the stacking gel (Table VIII) was loaded on the top of the resolving gel and left to polymerize, during 30 min at RT. The samples were boiled, during 5 min, in loading gel buffer (LB) to allow the protein denaturation. Precision plus protein standards Dual Color (BioRad) was used as markers. Proteins were separated at 90 mA, during approximately 3 hours in a Hoefer electrophoresis system.

Table VIII – Stacking and resolving gels compositions (for one SDS-PAGE system, with 1.5 mm of thickness). LGB: Lower Gel Buffer; UGB: Upper Gel Buffer; SDS: Sodium dodecyl sulfate; APS: Ammonium persulfate; and TEMED: Tetramethylethylenediamine.

Reagents	Stacking gel	Resolving gel		
	3.5%	**7.5%** (1)	**5%** (2)	**20%** (2)
Water	13.83 mL	33.42 mL	18.65 mL	7.2 mL
Acrylamide (29:1)	1.75 mL	11.25 mL	3.75 mL	15 mL
LGB (4X)	---	15 mL	7.5 mL	7.5 mL
UGB (5X)	4 mL	---	---	---
10% SDS	200 µL	---	---	---
10% APS	200 µL	300 µL	150 µL	150 µL
TEMED	20 µL	30 µL	15 L	16 L

(1) Resolving gel percentage used for conditioned media; **(2)**Resolving gel percentage used for cell lysates.

3.3.3 – Western Blotting analysis

Western blotting is the technique used for detection of specific proteins in complex samples, such as cell culture supernatants, cell lysates, or body fluids. In this technique, the proteins separated by electrophoresis are transferred to a solid membrane by the application of an electrophoretic field.

The *sandwich* was prepared with the gel, nitrocellulose membrane, 3 MM paper, two porous pad and two plastic supports. Then, the proteins were transferred at 200 mA for at least 16 hours. Finally, the membrane was removed from the *sandwich* and allowed to dry at RT.

This is a fast and efficient procedure that preserves the high-resolution separation of proteins by SDS-PAGE. Once at the membrane, proteins are suitable to detection by total protein staining or to label the proteins of the interest with specific antibodies (Figure 10).

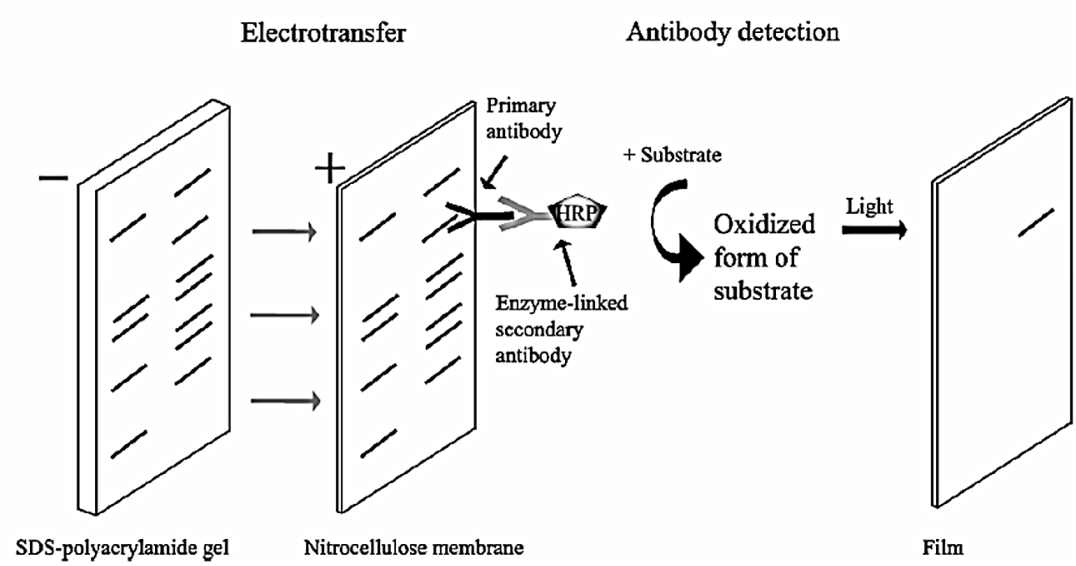

Figure 10 – Schematic illustration of the western blotting technique with indirect detection system using Enhanced Chemioluminescence (ECL). Proteins were separated using gel electrophoresis, transferred to a membrane and detected using labeled antibodies (adapted from [94]).

To detect the interest proteins by immunological and chemiluminescent protein detection were used a diversity of antibodies (Table IX and Figure 10).

The nitrocellulose membrane was hydrated with 1x TBS, during 5 min and blocked with 5% BSA in 1x TBS-T solution. This step ensures the blocking of possible non-specific binding-sites. After, membrane was incubated with an unlabeled primary antibody, directed against the target protein during 4 hours, with agitation at RT, following by an overnight (O.N.) incubation at 4ºC. The membrane was then washed with 1x TBS-T (3 times, 10 min each) and incubated with the secondary antibody, coupled with horseradish peroxidase (HRP) for 2 hours. Three additional washes were performed with 1x TBS-T before protein detection with the enhance chemiluminescence (ECL) reagent. This method is based on the oxidation of the cyclic diacylhydrazide luminal that results in light emission.

The membrane was incubated with the working mixture of chemiluminescent detection reagent (home-made ECL), during 1 minute, or with Luminata Crescendo for 5 min depending on the protein of interest (Table IX). The membrane was then exposed to autoradiography films (Kodak) in an X-ray film cassette. Films were exposed for different periods of time in order to optimize the signal, then developed and fixed with the appropriate solutions.

Table IX – Antibodies used to detect the proteins of interest and their detection methods. APP: Amyloid Precursor Protein; sAPP: soluble fragment APP; p-tau: phosphorylated tau.

Protein	1st Antibody	Species reactivity	2nd 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)	Luminata™ Crescendo
sAPP	Mouse anti-APP (N-Terminal 22C11). Dilution 1:250 (Boehringer)	Rat, human, monkey	Peroxidase labeled anti-mouse. Dilution 1:5000 (Amersham Pharmacia)	Luminata™ Crescendo
tau5	Mouse anti-Tau, clone tau-5 Dilution 1:500 (Millipore)	Bovine, human, mouse, rat, sheep	Peroxidase labeled anti-mouse. Dilution 1:5000 (Amersham Pharmacia)	ECL
p-tau (Ser396)	Rabbit anti-Tau (Phospho S396). Dilution 1:5000 (Abcam)	Mouse, rat, human	Peroxidase labeled anti-rabbit. Dilution 1:5000 (Amersham Pharmacia)	Luminata™ Crescendo

3.3.3.1 – Membrane stripping

To visualized different proteins with similar molecular weight (KDa), the blots were striped as following. First, a stripping solution PH 6.7 (contained Tris Base and SDS) was prepared and mixed with β-Mercaptoethanol. The nitrocellulose membranes were hydrated with 1x TBS and exposures to stripping solution, previously prepared, at 37ºC for 30 min with agitation. After, the stripping solution was discarded and the membranes washed 3 times with 1x TBS-T. Finally, membranes were washed with distillated water and dried at RT.

To confirm the stripping, membranes were rehydrated with 1x TBS and blocked with 5% BSA in 1x TBS-T solution, by 1 hour. Then, an incubation with the last used secondary antibody was made and the membranes developed with ECL reagent, Luminata Crescendo.

3.3.3.2 – Ponceau S staining

The nitrocellulose membrane was incubated in Ponceau S solution, during 5 min and washed with deionized water to make the bands visible. Then, the membrane was scanned in a GS-800 calibrated imaging densitometer and extensively washed with 1x TBS-T and deionized water to remove the staining. A region of the membrane was used as loading control.

3.3.3.3 – Quantitative analysis

The ImageLab software version 5.1 (BioRad) was used to quantify band intensities of the immunoblots and the Ponceau S staining.

3.4 – Statistical analysis

The software used for the statistical analysis was the GraphPad Prism version 4.0. Data was expressed as means ± S.D of three independent experiments (n).

Statistical significance analysis was conducted by ANOVA followed by Dunnett's test for multiple comparisons, where $P < 0.05$ was considered significant.

3.5 – Immunocytochemistry

The immunocytochemistry technique allows to detect and localize the antigens of interest in cells and tissues, using specific antibodies conjugated with a fluorophore.

Immunocytochemistry was performed to visualize the apoptotic nuclei as well as to detect the cytoskeletal proteins, namely acetylated α-tubulin and F-actin. Cells cultured on coverslips (15 mm) at a density of $1x10^5$ cells/well were fixed with 4% PFA for 20 minutes at RT. The fixing reagent was removed and cells were washed three times with 1x PBS. After, cells were permeabilized with 0.2% PBS-Triton for 10 minutes, washed three times with 1x PBS, and then blocked with 3% BSA in PBS for 1 hour at RT. At this stage, cells were also washed three times with 1x PBS.

The cells were then incubated with primary antibody for acetylated α-tubulin diluted in the blocking solution (1:250), during 2 hours, in humidified conditions. Cells were washed three times with 1x PBS and then incubated with the appropriate fluorescently-labelled secondary antibodies (Alexa Fluor 488, 1:300) in PBS with 0,3% BSA. In order to visualize F-actin, Alexa Fluor 568 Phalloidin was diluted (1:1000) in PBS with 0.3% BSA and added to coverslips for 1 hour in the dark at RT. Coverslips were further washed three times with 1x PBS and one time with distillated water. After, a drop of mounting medium (Vectashield with DAPI) was added to each slide for nuclei staining.

Fluorescence microscopy was performed using an IX81 Motorized Inverted Microscope (Olympus Corporation, Hamburg, Germany) equipped with an LCPlanFl 20x/0.40 objective lens. The analySIS 3.2 software (Olympus) was used for image acquisition of around 100 cells/condition and the ImageJ with a custom macro (Figure 11), to count the total nuclei. Apoptotic nuclei were counted manually and their percentage determined [(apoptotic nuclei/ total nuclei) * 100].

```
1  run("8-bit");
2  run("Unsharp Mask...", "radius=10 mask=0.90");
3  run("Median...", "radius=3");
4  run("Subtract Background...", "rolling=50");
5  setAutoThreshold("Default dark");
6  run("Threshold...");
7  setThreshold(40, 255);
8  setOption("BlackBackground", false);
9  run("Convert to Mask");
10 run("Make Binary");
11 run("Fill Holes");
12 run("Watershed");
13 run("Set Measurements...", "area redirect=None decimal=3");
14 run("Analyze Particles...","size=100-Infinity show=[Overlay Outlines] display clear summarize in_situ");
```

Figure 11 – List of commands contained in the ImageJ macro to count the total nuclei number.

LSM510 – Meta Confocal Microscope (Zeiss) with 63x/1.4 oil immersion objective and the Zeiss LSM510 4.0 software were used to visualize the cytoskeletal proteins. The argon laser lines of 405 nm, 488 nm, and a 561 nm DPSS laser were the wavelengths selected to acquire the confocal images.

In both cases, the setting used were the same for each condition and for each independent experiment (n).

4 – Results

4 – Results

4.1 – Evaluation of IL-8 and MCP-1 cytotoxicity

Inflammation is a key event in AD pathogenesis. Several inflammatory mediators have been shown altered in AD patients. Chronic inflammation can result in subsequent apoptosis and contribute to neurodegeneration typical of AD. To date little is known about chemokines in particular MCP-1 and IL-8 on AD. Hence the first step of this thesis was to determine the impact of these chemokines on cell toxicity. For that SH-SY5Y neuroblastoma cells were differentiated with retinoic acid (RA) and incubated with different concentrations of IL-8 and MCP-1 at different time periods, as explained in the sections 3.1.1 and 3.1.2, respectively. Following the differentiation period, cell morphological alterations were visible, e.g. long extensions (Figure 11A). Microtubule-associated protein 2 (MAP-2) and β3-tubulin were also used as markers of cell differentiation (data not shown). SH-SY5Y cellular viability was measured using the MTT assay. Cells were incubated in free serum culture medium with MTT (Cf = 0.5 mg/ml) during 4 hours at 37ºC, and the precipitates (dark purple) resulting from mitochondrial reduction of MTT to formazan, dissolved with DMSO, and absorbance read at 570 nm. Since reduction of MTT can only occur in metabolically active cells, the level of activity served as a measure of cell viability. Experiments carried out showed that cells exposed to IL-8 during 48 hours, exhibited more dead cells floating in the culture medium, when compared with untreated control cells (Figure 11E). Despite that, cells were not significantly affected, as observed even for the higher concentration, 150 ng/mL. Interestingly, a significant increase (approximately 25%) could be observed for the 10 ng/mL concentration (Figure 11F).

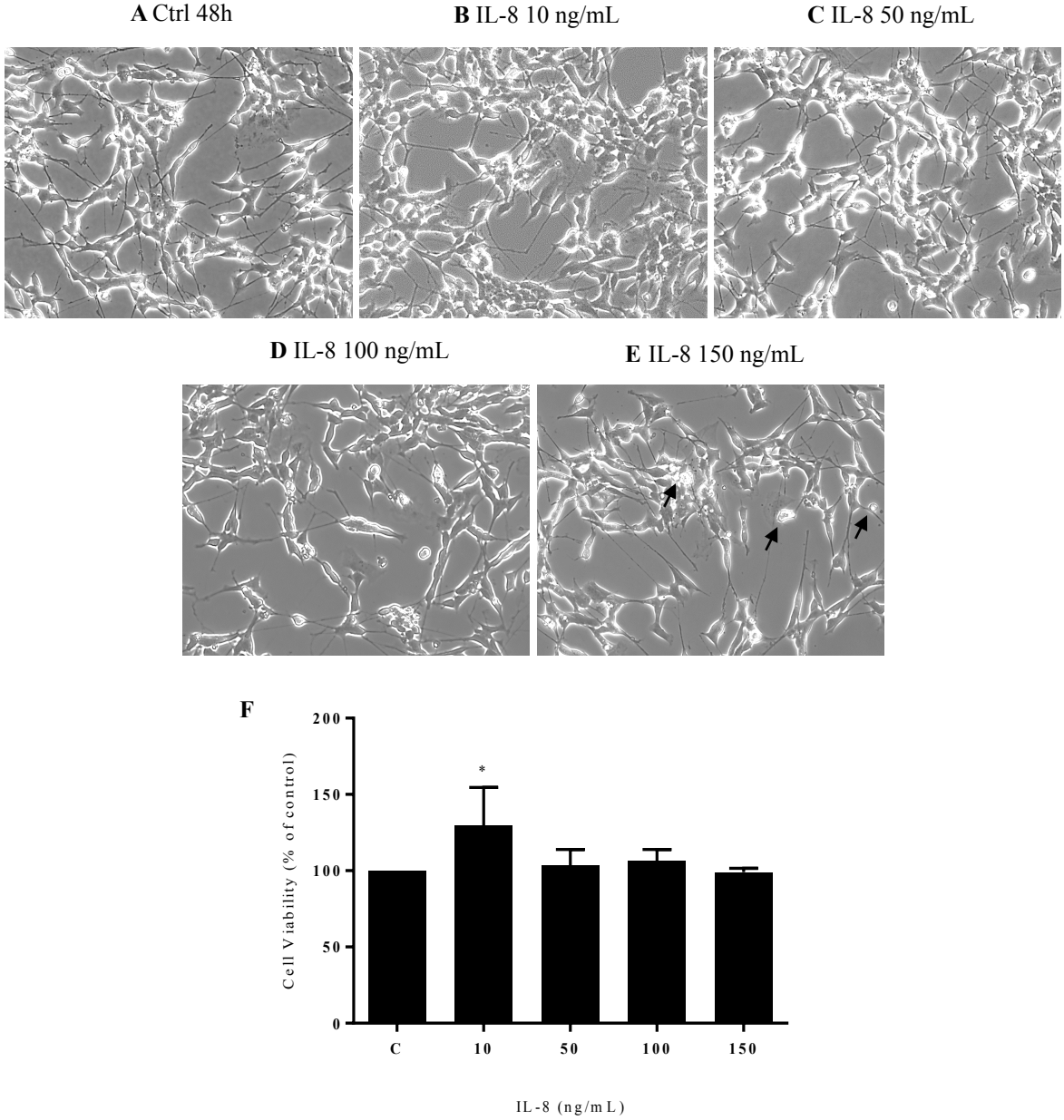

Figure 11 – IL-8 induced cytotoxicity in SH-SY5Y differentiated cells. Untreated control cells **(A)** and cells incubated with IL-8 during 48 hours, at 10 (**B**), 50 **(C)**, 100 **(D)** and 150 **(E)** ng/mL. At higher concentrations, such as 150 ng/mL, it is possible to visualize more dead cells (black arrows) floating in the culture medium. A magnification of 200x is shown. **(F) IL-8 effects in cellular viability.** Cell viability was measured for the incubation of 48 hours, using the MTT assay and the results were expressed as % of control. An increase with statistical significance was observed for the concentration of 10 ng/mL, with *P<0.05 by Dunnett's test. All values are expressed as mean ± SD from three independent experiments.

Similar data were obtained when cells were incubated with MCP-1 at different concentrations for 24 hours. The appearance of the SH-SY5Y cells after incubation with MCP-1 at 150 ng/mL resembles to the one seen for IL-8 at 150 ng/mL, with some round and dead cells floating in the culture medium (Figure 12E). However, no significant

differences were observed for the cellular viability when cells were incubated with MCP-1. Only a slight decrease in the percentage of viable cells for the higher concentration of the MCP-1, could be observed (around 10% bellow control cells), Figure 12F.

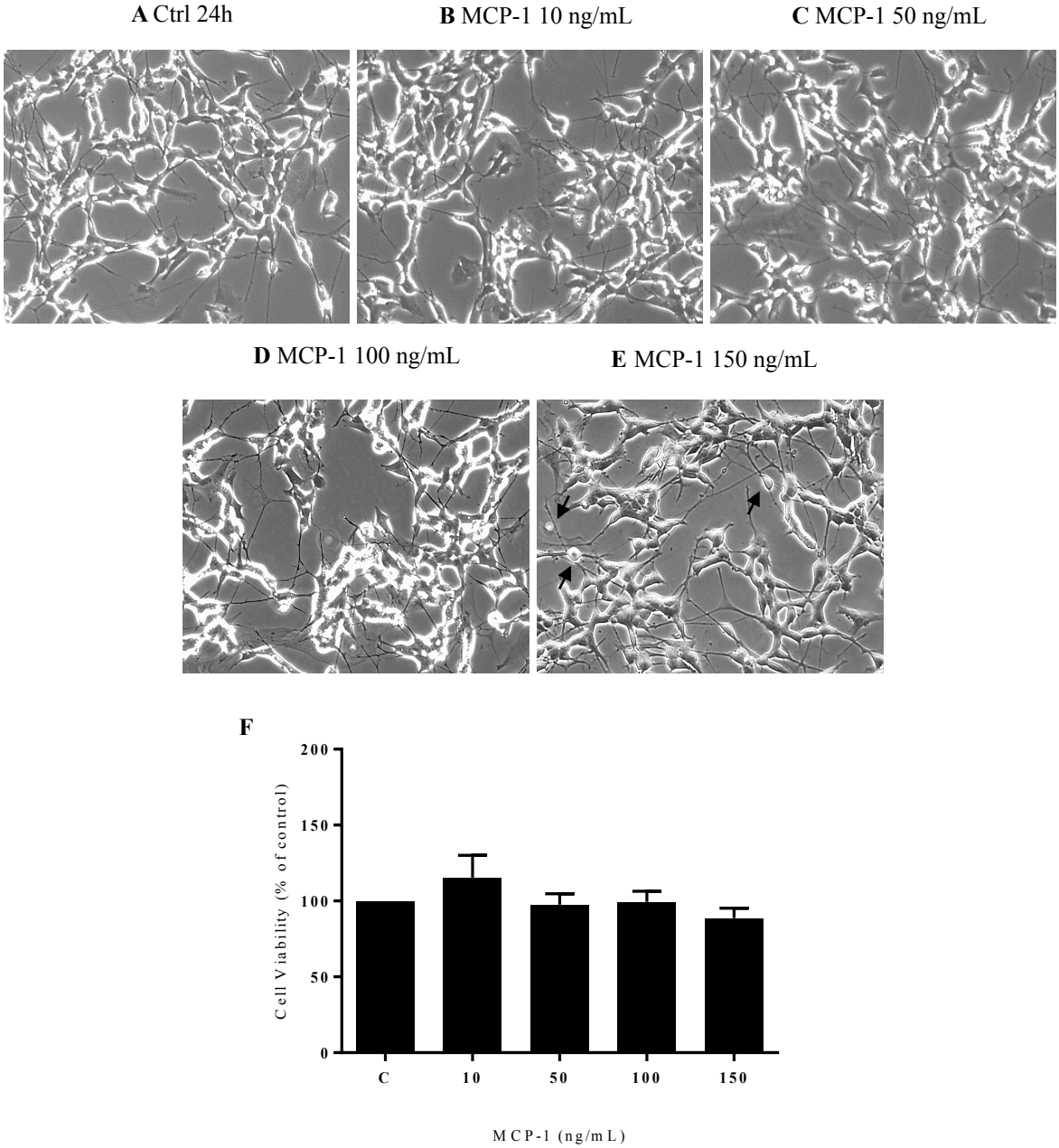

Figure 12 – MCP-1 cytotoxic effects in SH-SY5Y differentiated cells. (A) Untreated control cells and cells incubated with MCP-1 during 24 hours, at 10 **(B)**, 50 **(C)**, 100 **(D)** and 150 **(E)** ng/mL, seen at a magnification of 200x. For the higher concentration (150 ng/mL) more rounded and dead cells (black arrows) were visualized. **(F) MCP-1 effects in cellular viability.** Cell viability was measured for the incubation of 24 hours, using the MTT assay and the results were expressed as % of control. For the MCP-1 at 150 ng/mL lower cell viability relative to control was seen. All values are expressed as mean ± SD from three independent experiments.

In parallel, the cytotoxic effects of the chemokines were further addressed using immunofluorescent studies. Differentiated SH-SY5Y were exposed to IL-8 and MCP-1, and cell nuclei stained as described in section 3.5 to evaluate apoptosis. The nuclei were stained with DAPI, which binds strongly to A-T rich regions in DNA emitting blue fluorescence. An ImageJ macro was developed to count the nuclei in each of the acquired images by fluorescence microscopy. Consistently with MTT data, no significant differences could be detected in IL-8 treated cells, when compared with the untreated control cells. However, a tendency to increase in the number of apoptotic nuclei was observed at the higher IL-8 concentration for the longer period, but not statistically significant (Figure 13F).

During apoptosis, the chromatin suffers a change from a heterogeneous and genetically active network, to an inert highly condensed form whose fragments are packaged into apoptotic bodies [95]. Membrane blebbing (Figure 13E), which occurs due to a contractile force generated by actin-myosin cytoskeletal structures [96] and cell shrinkage (Figure 14E), are typical features of apoptotic cells both observed in IL-8 and MCP-1 treated cells.

Relatively to the cells incubated with MCP-1 for 24 hours, differences can be observed regarding to percentage of apoptotic nuclei. It was obtained a significant increase in about 5% of apoptosis for the concentration of 150 ng/mL, when compared with the control cells, as seen in the Figure 14F.

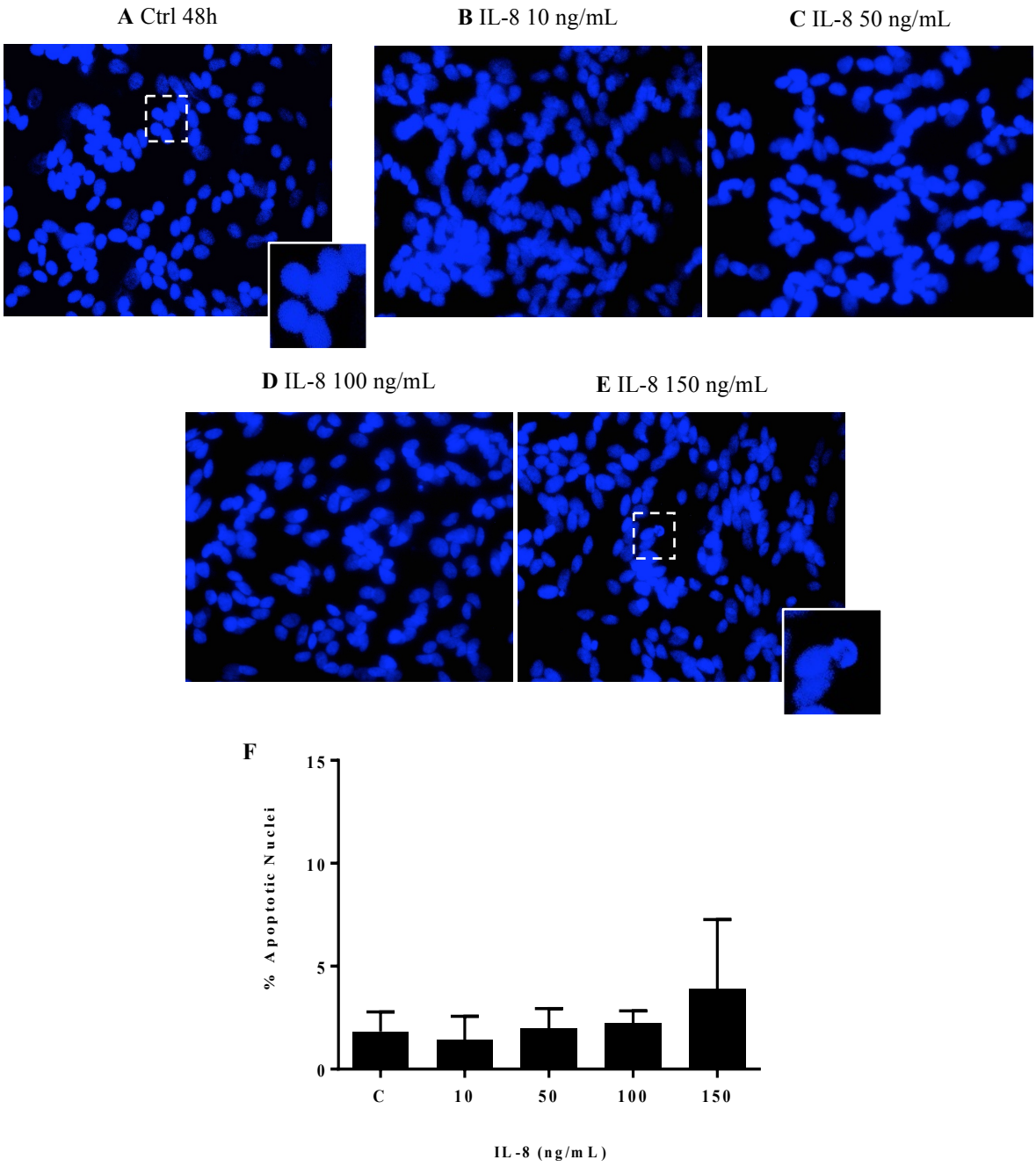

Figure 13 – IL-8 apoptotic effects on differentiated SH-SY5Y cells. Apoptotic nuclei fluorescence images **(A-E)** and the respective number obtained for each concentration at 48 hours **(F)**. Differentiated untreated control cells **(A)** and cells incubated with IL-8 at 10 **(B)**, 50 **(C)**, 100 **(D)** and 150 **(E)** ng/mL. In **A** and **E** is shown a zoomed image where it is possible to visualize normal and apoptotic nuclei, respectively. A magnification of 200x was used during the image acquisition and to each condition, the apoptotic nuclei relatively to total nuclei (% of the apoptotic nuclei) was determined. All values are expressed as mean ± SD from three independent experiments.

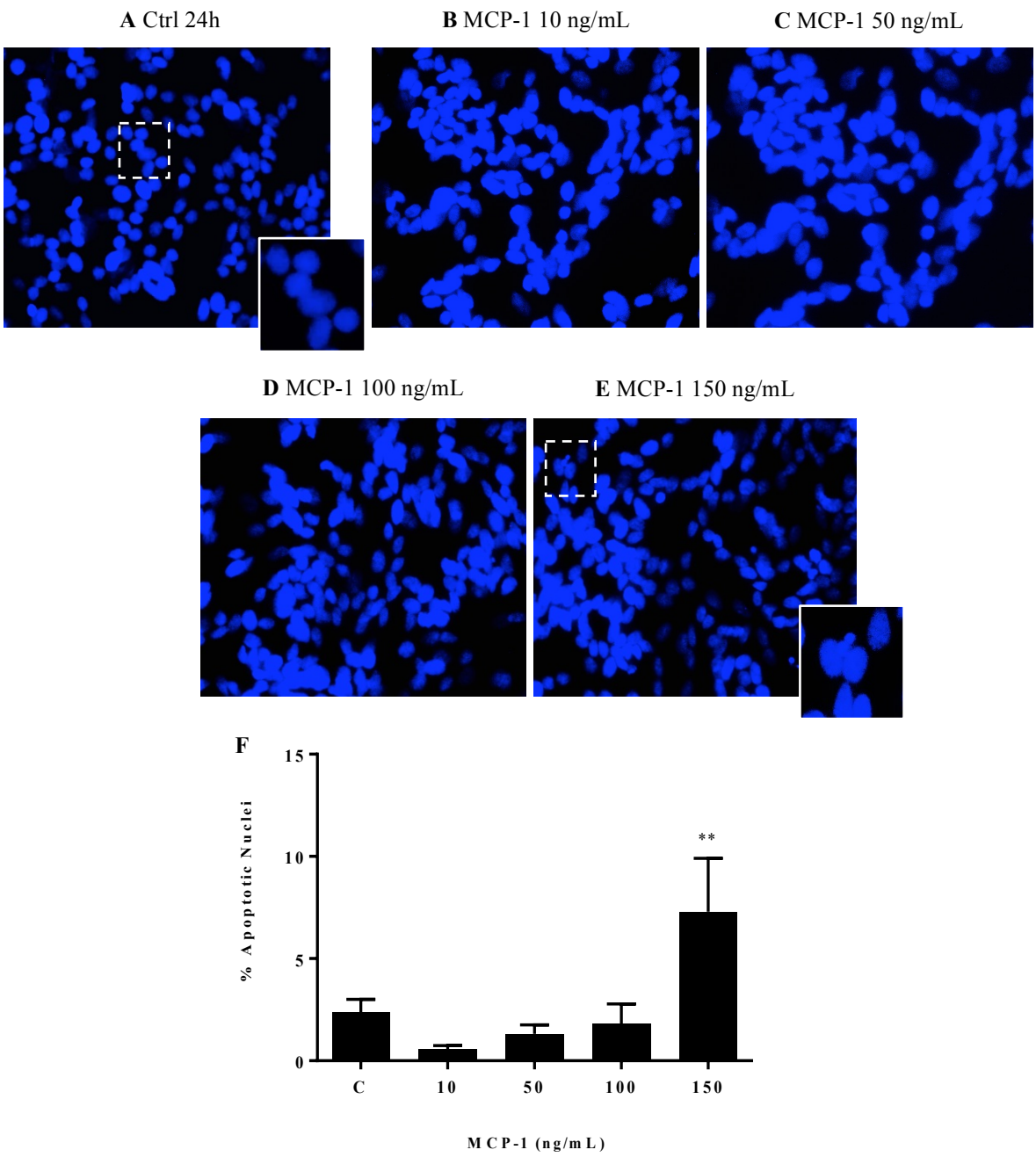

Figure 14 – MCP-1 apoptotic effects on differentiated SH-SY5Y cells. Apoptotic nuclei fluorescence images **(A-E)** and the respective count obtained for each concentration at 24 hours **(F)**. Differentiated untreated control cells **(A)** and cells incubated with MCP-1 at 10 **(B)**, 50 **(C)**, 100 **(D)** and 150 **(E)** ng/mL, observed at a 200x magnification. In **A** and **E** is shown a zoomed image where it is possible to visualize normal and apoptotic nuclei, respectively. To each condition, the apoptotic nuclei relatively to total nuclei (% of the apoptotic nuclei) was determined and for MCP-1 at 150 ng/mL, a statistically significant increase in the apoptotic nuclei number was observed, when compared with the untreated control cells, with **P<0.01 by Dunnett's test. All values are expressed as mean ± SD from three independent experiments.

In summary, and under the conditions tested both IL-8 and MCP-1 chemokines had little effects on MTT viability and apoptosis in SH-SY5Y differentiated cell line.

4.2 – IL-8 and MCP-1 effects on APP processing

To date, few studies had focused on the effects of chemokines on APP processing and Aβ production, and in particular, little is known about IL-8 and MCP-1 effects on APP processing.

In order to evaluate how these chemokines affect APP processing, differentiated neuroblastoma cells were treated with IL-8 at different concentrations (10, 50, 100, 150 ng/mL) for 24 and 48 hours. After the appropriated incubation period, cell lysates and conditioned media were collected and respectively probed with specific antibodies for the protein of interest. The rabbit polyclonal antibody anti-APP (APP C-Terminal) was used to detect full-length APP in the cell lysates, and the mouse monoclonal anti-APP (22C11, N-Terminal) was used to recognize the APP N-terminal fragments (sAPP) in the conditioned medium.

Upon IL-8 incubation for 24 and 48 hours, no significant differences could be detected for APP levels and sAPP fragment (Figure 15). Despite that, in both cases at 48 hours a tendency to increase relatively to the shorter incubation period could be observed (Figure 15).

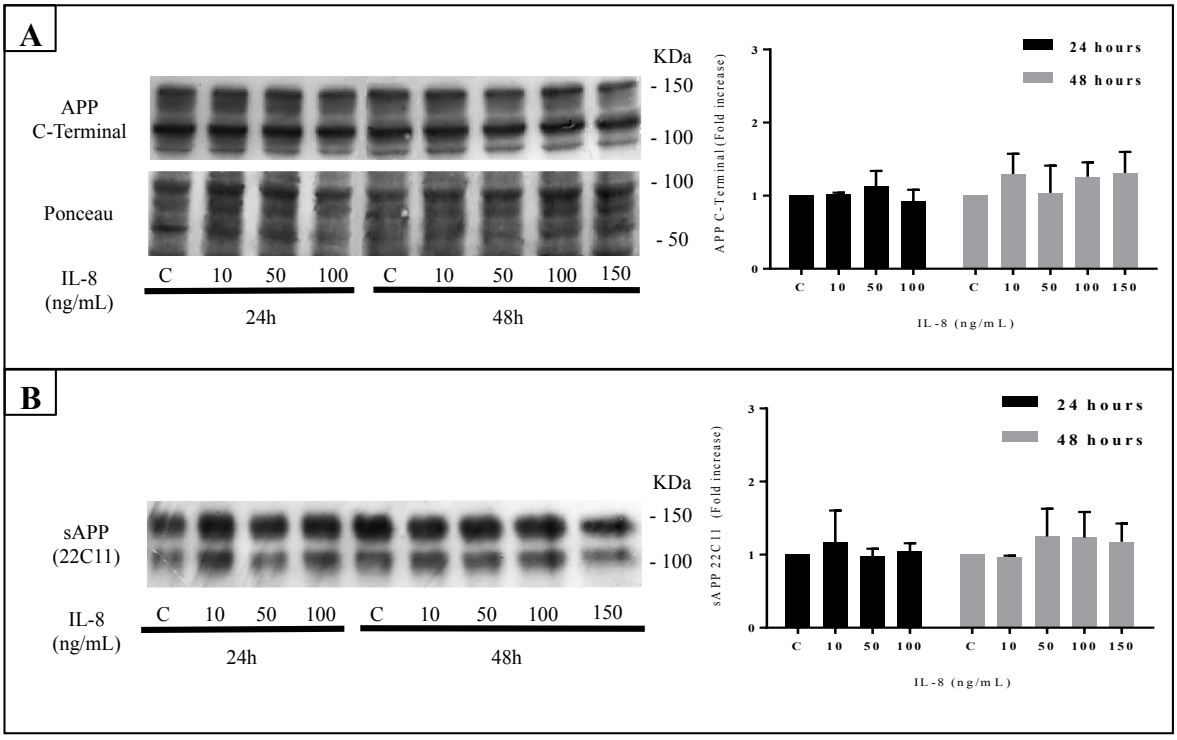

Figure 15 – IL-8 effects on APP processing on differentiated SH-SY5Y cells. Cells were differentiated and treated with IL-8 during 24 and 48 hours after which, cell lysates and conditioned media were collected and probed with APP C-Terminal **(A)** and APP N-Terminal 22C11 **(B)** antibodies. Quantification of APP levels by densitometric scanning was normalized to protein levels determined by Ponceau S staining. All values are expressed as mean ± SD from three independent experiments.

For MCP-1 chemokine, again a tendency to increase for APP C-Terminal could be detected after 6 hours of incubation, however at 24 hours, for the APP levels no differences could be observed (Figure 16A). These results were accompanied by no significant variations on sAPP secretion, as detected by the APP N-Terminal 22C11 antibody (Figure 16B).

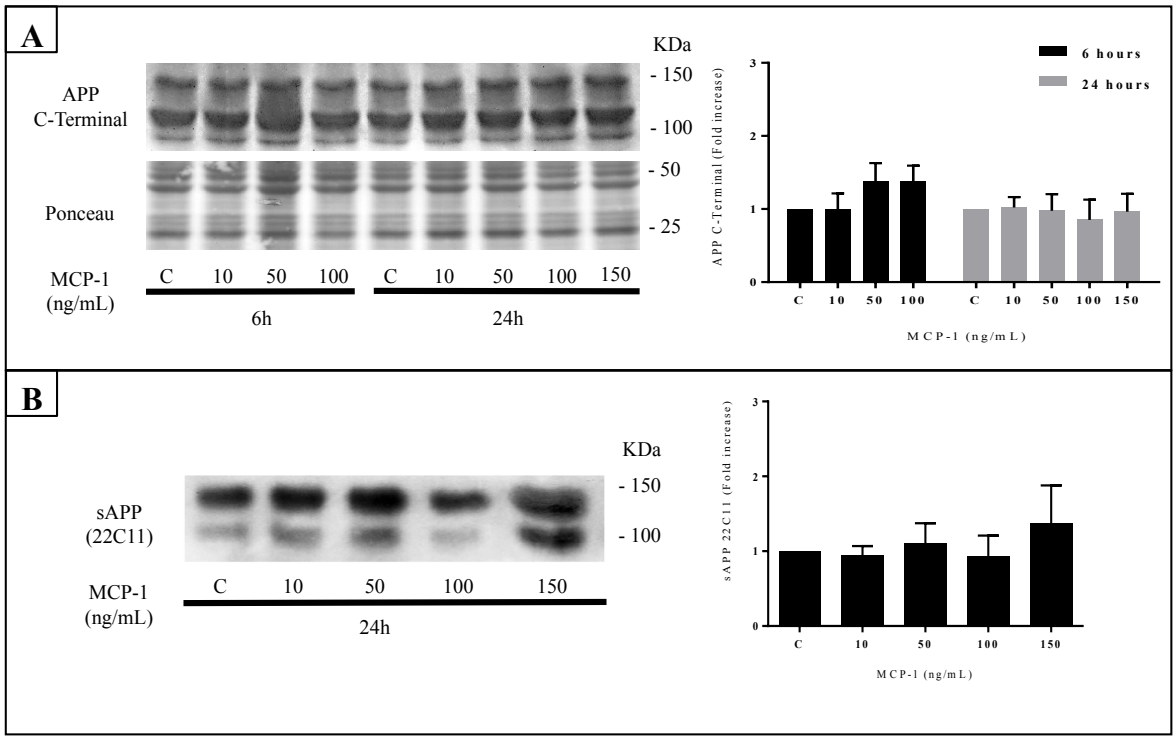

Figure 16 – MCP-1 effects on APP processing on differentiated SH-SY5Y cells. Cells were differentiated and treated with MCP-1 during 6 and 24 hours and then, cell lysates and conditioned media were collected and probed with APP C-Terminal **(A)** and APP N-Terminal 22C11 **(B)** antibodies respectively. Quantification of APP levels obtained by densitometric scanning was normalized to protein levels determined by Ponceau S staining. Since the sAPP levels were difficult to detect at 6 hours of incubation, only the results for the 24 hours are shown. All values are expressed as mean ± SD from three independent experiments.

Similar data were obtained for undifferentiated SH-SY5Y cells (Figure 17).

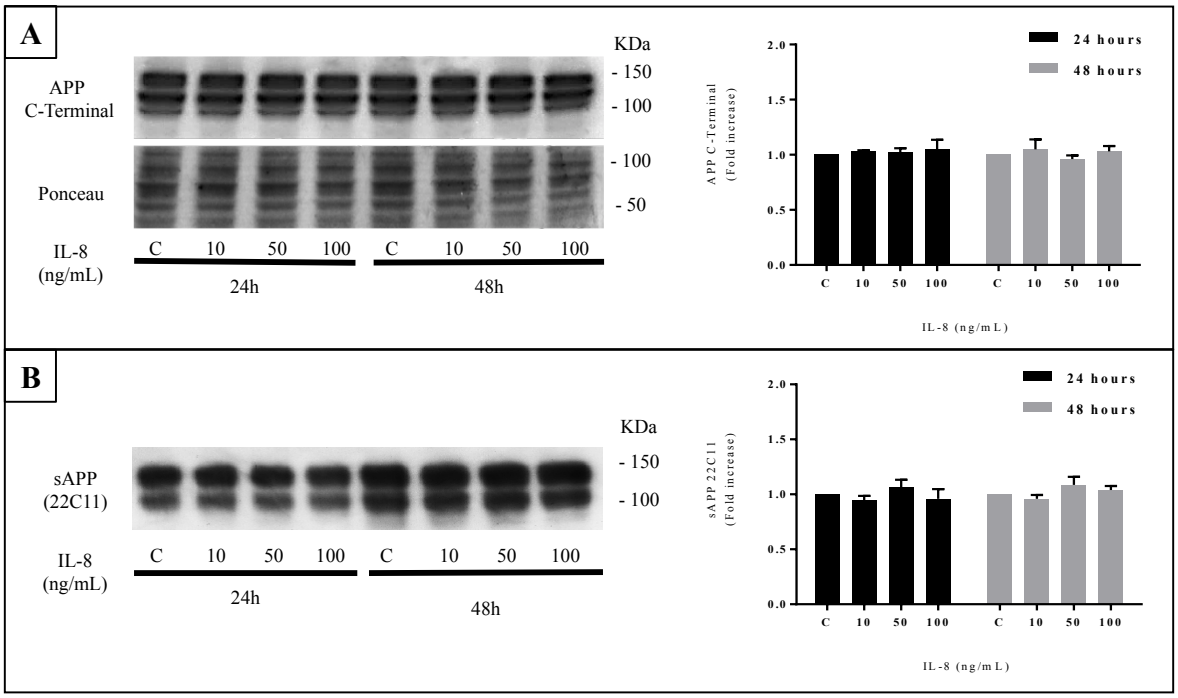

Figure 17 – IL-8 effects on APP processing. After Il-8 treatment during 24 and 48 hours, SH-SY5Y cell lysates and conditioned media were collected and probed with APP C-Terminal **(A)** and APP N-Terminal 22C11**(B)** antibody. Quantification of APP levels by densitometric scanning was normalized to protein levels determined by Ponceau S staining. All values are expressed as mean ± SD from three independent experiments.

Taken together, and although some tendency to increase could be observed, data suggests that these chemokines had no significant impact on APP processing in SH-SY5Y differentiated cells.

4.3 – Impact of the chemokines on tau and other cytoskeleton-associated proteins

As mentioned, Alzheimer's disease pathogenesis is associated with changes at the cytoskeletal level, namely on tau protein, a cytoskeletal protein than can be hyperphosphorylated and lead to NFT formation, a typical AD hallmark. Hence, the effects on tau and on its phosphorylated state were evaluated.

Differentiated SH-SY5Y cells were incubated with IL-8 and MCP-1 at 10, 50, 100 and 150 ng/mL, during 48 and 24 hours respectively. Cell lysates were collected and probed for t-tau and p-tau, in particular the Ser396 residue.

As indicated in Figure 18A, when differentiated SH-SY5Y cells were incubated with increasing concentration of IL-8 the levels of t-tau detected with the Tau5 antibody did not fluctuate markedly. In contrast at the concentration of 150 ng/mL an increase in the p-tau (Ser396) was detected. In order to have a comparable term of tau phosphorylated state in response to IL-8, the ratio of p-tau (Ser396) versus t-tau was calculated. A tendency to increase in p-tau could be observed on particular for the 150 ng/mL of IL-8 by approximately 1.2 fold (Figure 18A).

Relatively to MCP-1 (Figure 18B) similar data was obtained with the observation of 1.4 fold increase on t-tau versus p-tau (Ser396) ratio at 100 ng/mL. This increase was not sustained at the highest MCP-1 concentration when compared to control cells.

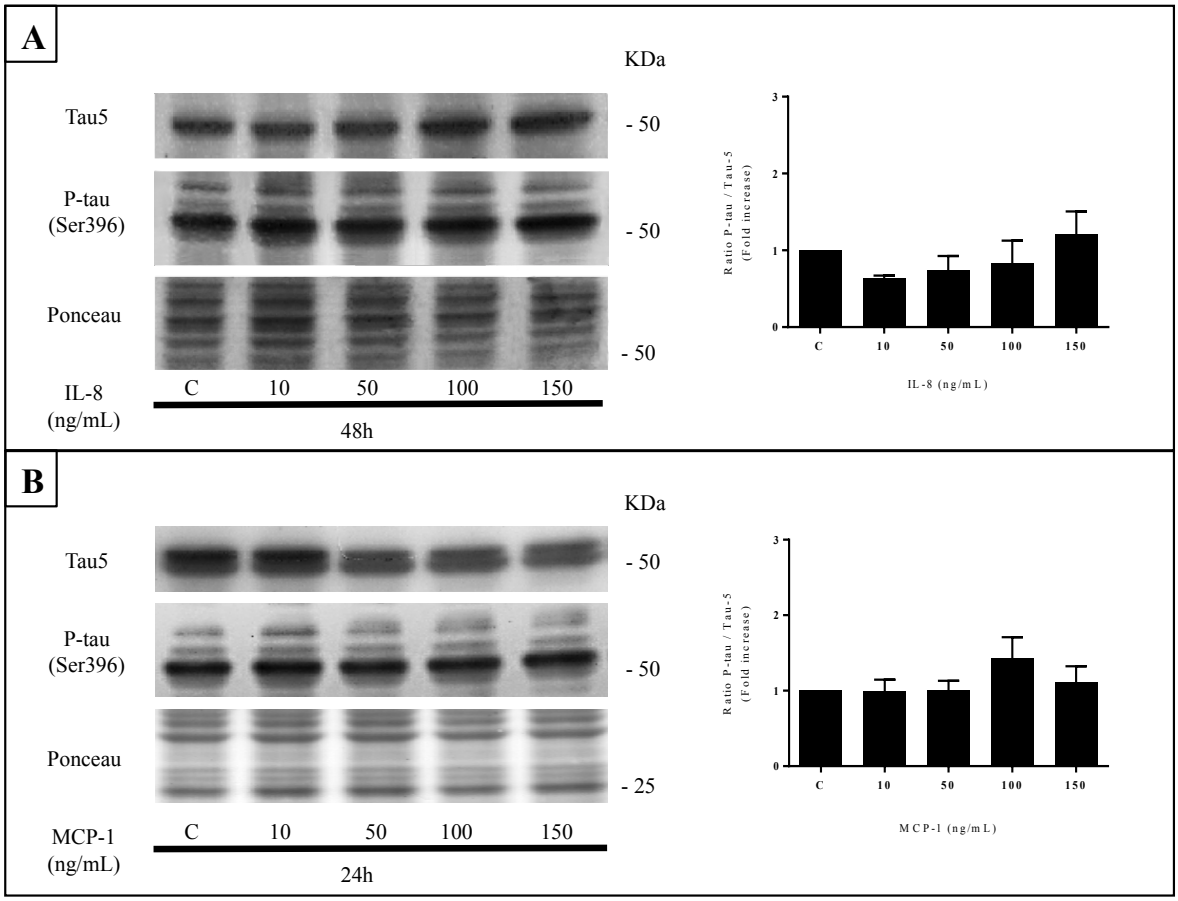

Figure 18 – Chemokines impact on tau phosphorylation. P-tau/Tau-5 ratio after SH-SY5Y cells differentiation and treatment with IL-8 for 48 hours **(A)** and MCP-1 for 24 hours **(B).** Cell lysates were collected and probed with p-tau (Ser396) and Tau-5 antibodies. Graphs depict tau phosphorylated levels at the Ser396 residue with values normalized for t-tau (Tau5) content. All values are expressed as mean ± SD from three independent experiments.

The effects of these chemokines on cytoskeleton were further explored by evaluating the impact of IL-8 and MCP-1 on actin and tubulin networks. In particular, F-actin polymerization and α-tubulin acetylation in the presence or absence of these chemokines were monitored. Under control conditions, some actin fibres could be observed but this appeared shorter and thinner when compared to IL-8 treated cells. In contrast, for MCP-1 there was a decrease on F-actin filaments comparatively to control cells (Figure 19). Of note, for both chemokines an increase on α-tubulin acetylation at the highest concentration (150 ng/mL) was observed, compared to untreated control cells (Figure 19).

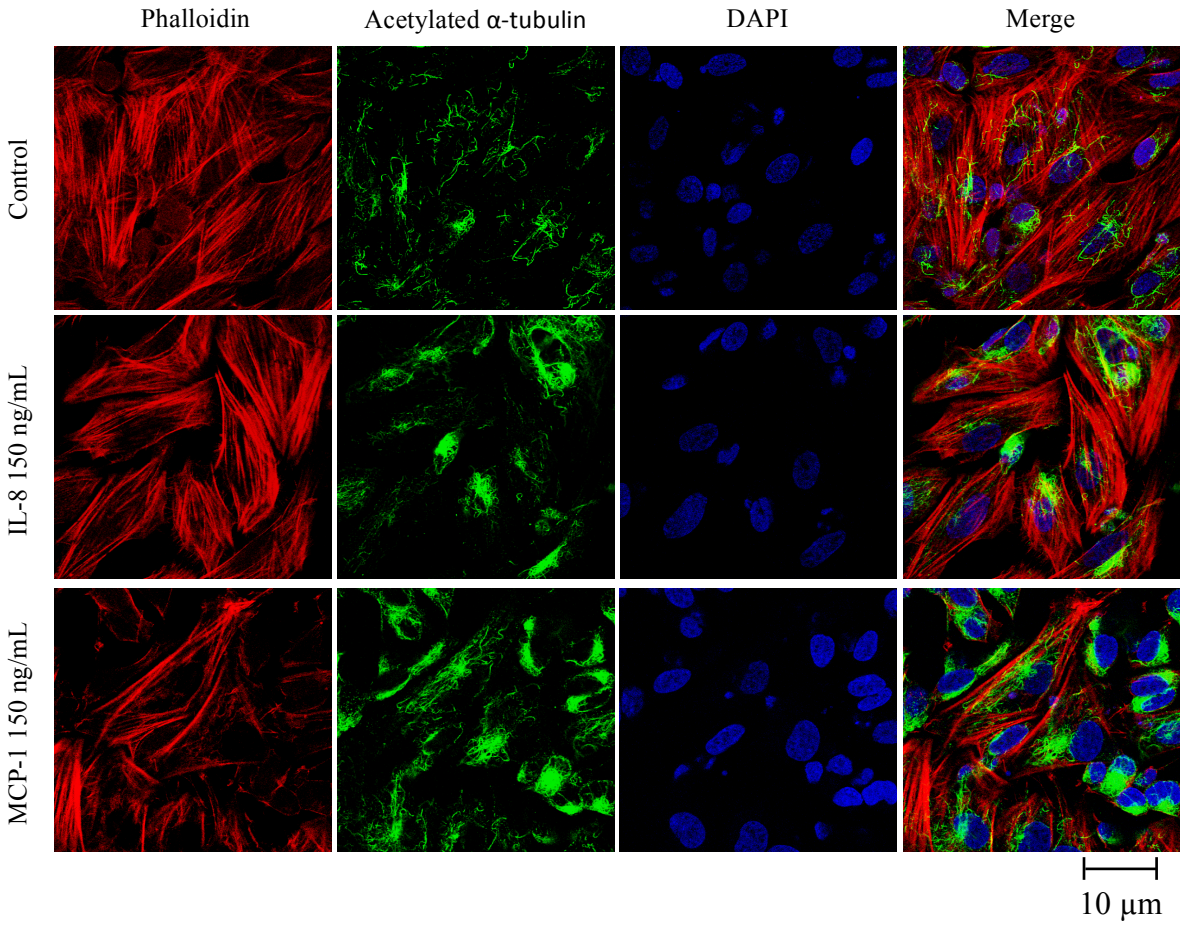

Figure 19 – The effects of chemokines on cytoskeleton network. Upon differentiation and chemokine treatments, SH-SY5Y cells were stained and both actin and tubulin networks evaluated. Two antibodies were used to stain the cells: Alexa Fluor 568 conjugated phallotoxin solution (labelled filamentous F-actin) and acetylated α-tubulin antibody with fluorescently-labelled secondary antibody (Alexa Fluor 488). The nuclei were stained with DAPI and during image acquisition, a 63x/1.4 oil immersion objective was used. Images were acquired using a LSM510 - Meta Confocal Microscope.

Overall, our data indicate that under the conditions tested, MCP-1 and IL-8 had impact on cytoskeleton network. However additional studies are needed to clarify these effects and to unveil the underlying molecular mechanisms.

5 – Discussion

5 – Discussion

Alzheimer's disease is the most common type of dementia, characterized by progressive cognitive decline, usually beginning with impairment in the ability to form recent memories and then, affecting all intellectual functions, leading to premature death.

The inflammatory process plays an important role in AD pathogenesis, with signs of chronic neuroinflammation and altered levels of some cytokines being linked to the disease. The presence of inflammatory molecules is likely to be a very early event in AD pathogenesis, preceding the clinical onset of the disease. Many studies have focused on the potential of the inflammatory molecules as candidate biomarkers for AD diagnosis, however many of these data are still controversial and inconclusive. This work aimed to contribute to the understanding of the role of chemokines, such as IL-8 and MCP-1 on AD. Namely, this study addressed their apoptotic properties and impact on APP and tau, two key proteins in AD pathogenesis.

IL-8 and MCP-1 cytotoxicity

According to Thirumangalakudi *et al* [91], IL-8 may have two distinct effects on neuronal cells associated with a dose-dependent manner. At higher concentrations of this chemokine there was a significant decrease in cell survival. Nevertheless, at low concentrations IL-8 appears to have a neurotrophic effect, registering its peak at 10 ng/mL with a registered survival of 120%. This data is consistent with our own results, where at a concentration of 10 ng/mL IL-8 leads to a 25% of cell viability, as determined by MTT (Figure 11). No significant decrease of cell viability or apoptosis was observed for this chemokine, under the tested conditions (Figure 13). Despite that, the same study [91] reported that neuronal exposure to IL-8, leads to a dose-dependent expression of Bim, implicating this protein as an important mediator of the IL-8 neuronal cell death cascade.

With respect to the MCP-1 and its cytotoxicity, a more evident decrease to approximately 90% of viable cells was obtained, mainly at the concentration of 150 ng/mL (Figure 12). Yamamoto *et al* [88] tested the effects of MCP-1 in PC12 cells on Aβ concentration, when cells were treated for 24 hours. Data showed a decrease in this peptide at the 10 ng/mL concentration of MCP-1. This may correlate with the increase in cell survival verified in our experiments for the same concentration.

Regarding to the results obtained for the apoptosis, a significant difference was detected in the number of apoptotic nuclei for the highest MCP-1 concentration (150 ng/mL). This points in the same direction as the MTT assay, where lower cell viability corresponds to a high number of apoptotic nuclei.

Yang *et al* [97] reported that in a neuron/microglia co-culture with a previous induced damage, the addition of MCP-1 increment the neuronal death. It is known that under an inflammatory response, microglia is activated and releases some inflammatory mediators, in which MCP-1 is included. MCP-1 release may affect neuronal cells viability and death contributing to AD neurodegeneration.

IL-8 and MCP-1 on APP

Additional studies were directed for the effects of MCP-1 and IL-8 on both APP and tau. These two proteins are in the basis of Aβ production or NFT formation.

IL-8 is a chemokine produced by macrophages in the presence of the Aβ peptide [48], being important for activated microglia recruitment to the sites of the damaged brain. To date, there is no information about the IL-8 effects on APP processing and Aβ production. This work focused on the evaluation of the IL-8 effects on APP itself and on sAPP secretion, in a neuronal model of SH-SY5Y differentiated cells. No significant differences were detected in APP levels, although for the longer period a tendency to increase on sAPP secretion and APP were observed, when compared to the shorter incubation period (Figure 15).

MCP-1 is a chemokine produced by microglial cells and stimulates astrocytes, which together can participate in the degradation of the Aβ peptide. However, when MCP-1 is overexpressed, it induces microgliosis and enhances Aβ deposits as well as its uptake by the microglia. This uptake promotes the oligomerization of Aβ species in both the intracellular and extracellular fractions. Lack of available information for MCP-1 and APP processing leads us to focus on this chemokine.

Like for IL-8, when SH-SY5Y cells were incubated with MCP-1 no significant differences could be observed. Only a marginal increase on full-length APP could be detected for the lower incubation period, as well as for the sAPP levels, mainly at the highest concentration (150 ng/mL) for 24 hours (Figure 16).

Although change in the levels of those chemokines have been reported in AD, and not excluding other roles of this in the disease, we can suggest that the two tested

chemokines produce marginal changes on APP metabolism, based on the data obtained. Additional set of experiments only could be carried out to certify the data and minimize the SD variation, leading to a more accurate correlation between these chemokines, APP processing and the Alzheimer's disease pathogenesis.

Chemokines impact on tau and other cytoskeletal proteins

Tau is a microtubule-associated protein that helps in the microtubular and cytoskeletal stability. Alterations in this protein leads to NFT formation and abnormal axonal transport with subsequent energetic and nutritional deficit rendering in neuronal death [6]. For IL-8 our experiments revealed a decrease in tau phosphorylation relatively to the total tau at the lower concentration, and an increase at the concentration of 150 ng/mL. Interestingly the decrease on p-tau correlates with an increase on cell viability for IL-8 at 10 ng/mL (Figures 18A and 11B).

For MCP-1 a tendency to increase on p-tau (Ser396) residue could be observed at the higher concentration (Figure 18B). Like for APP processing, no significant differences could be observed for these chemokines regarding the phosphorylation of the Ser396 residue. However other relevant residues in AD perspective, could be addressed, namely Ser202 and Ser262 [98] whose phosphorylation are involved in the formation of pre-NFT, controlling the microtubule-binding ability as well as the axonal transport regulation [99,100].

Many cytoskeletal abnormalities have been reported in AD not only on tau but also at the actin and tubulin level [101]. Actin filaments in the cell cortex determine the shape, stiffness and the movement of the cell surface. These are highly dynamic proteins, whose polymerization is usually correlated with their disassembly [102]. In particular for IL-8 treated cells, some bundles of actin filaments were seen, which suggests an increase in F-actin polymerization with possible consequences to the cell dynamic and integrity.

Tubulin is a protein that composes the cytoskeletal microtubules responsible for the cell shape, transport, motility and division. Studies have shown that alterations on tubulin acetylation can trigger cell death, possibly linked to the Alzheimer's disease pathogenesis [101,103]. Interestingly, data obtained for both chemokines show an increase in acetylated α-tubulin upon cell incubations with IL-8 and MCP-1, that appears to be more marked for the last one (Figure 19). Additional studies should be carried out in order to address

cytoskeleton signal molecules that could be involved in these effects, for e.g. Rac and others.

Zhang *et al* [104], refers that there is a compensatory mechanism between tau phosphorylation and α-tubulin acetylation. This occurs when tau stabilizing activity on the cytoskeletal microtubules is lost and the α-tubulin acetylation raises to maintain the balance. Data obtained for IL-8 is in agreement with this observation, since that with the increase of the p-tau (Ser396), an increase in α-tubulin acetylated was detected (Figures 18A and 19).

IL-8 and MCP-1 showed to change the cytoskeleton-proteins dynamics, however additional studies can improve knowledge behind mechanisms of these chemokines on cytoskeleton networks.

Conclusion & Future Perspectives

IL-8 and MCP-1 are two chemokines known to be altered in AD patients. However the role of these chemokines on AD pathogenesis is little explored. The work developed in this thesis, reveal that the tested chemokines had little effects on APP and tau, only showing marginal increase on apoptosis, APP processing and tau phosphorylation. Interestingly, effects on both actin and tubulin networks were observed. Presently it is difficult to interpret the data obtained since little is known about the mode of action of these chemokines. Complementary studies should be performed, not only to evaluate other APP fragments resulting from its processing, such as the Aβ peptide itself, but also to clarify the IL-8 and MCP-1 effects on the cytoskeleton-associated proteins. Studies including cytoskeleton modulating drugs or specifically addressing signal molecules involved in cytoskeletal dynamics, could aid in the understanding of the mechanisms underlying these effects. In sum, from the work herein presented, the most prominent effect of these chemokines was at the cytoskeletal level. Cytoskeletal alterations have been associated with AD pathogenesis by impacting on neuronal dynamics and survival, consequently leading to neurodegeneration.

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7 – Annexes

7 – Annexes

In this section is listed the equipment and the solutions used for the experiments developed in this thesis.

7.1 – Cell Culture

Equipment:

- Hera cell CO2 incubator (Heraeus)
- Safety cabinet Hera safe (Heraeus)
- Inverted optical microscope (LEICA)
- Hemacytometer (Sigma)
- Sonicator (U200S IKA)
- Bath SBB6 (Grant)
- Culture Plates (Corning)

Reagents and Solutions:

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

MEM (Gibco, Invitrogen)	4.805 g
F12 (Gibco, Invitrogen)	5.315 g
$NaHCO_3$ (Sigma)	1.7 g
Sodium Pyruvate (Sigma)	0.055 g
1% Antibiotic/Antimycotic (AA) mix (Gibco, Invitrogen)	10 mL
10% FBS (Gibco, Invitrogen)	100 mL
L-Gluatamine (200 mM stock solution)	2.5 mL

- **Differentiation medium 1% FBS MEM:12 (1:1)**

MEM (Gibco, Invitrogen)	4.805 g
F12 (Gibco, Invitrogen)	5.315 g
$NaHCO_3$ (Sigma)	1.7 g

Sodium Pyruvate (Sigma)	0.055 g
1% Antibiotic/Antimycotic (AA) mix (Gibco, Invitrogen)	10 mL
1% FBS (Gibco, Invitrogen)	10 mL
L-Gluatamine (200 mM stock solution)	2.5 mL
Retinoic Acid (20 mM stock solution)	500 µL

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 4ºC.

- **PBS (1x)**

Dissolve one sack of BupH Modified Dulbecco's Phosphatase Buffered Saline Pack (Pierce) in deionized H_2O.

Final composition:

Sodium Phospatase	8 mM
Potassium Phosphatase	2 mM
Sodium Chloride	140 mM
Potassium Chloride	10 mM

Sterilize by filtering through a 0.2 µm filter and store at 4ºC.

- **RIPA Buffer (Sigma-Aldrich)**

For 6.5 mL of RIPA buffer add:

NaF	40.3 µL
NaO	65 µL
Protease Inhibitor cocktail (Sigma-Aldrich)	65 µL

7.2 – MTT assay

Equipment:

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

Reagents and Solutions:

- MTT (0.5 mg/mL) (Sigma-Aldrich)
- DMSO (99.9%) (Fisher Scientific)

7.3 – Protein Content Determination

Equipment:

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

Reagents and 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.

7.4 – SDS-PAGE

Equipment:

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

Reagents and Solutions:

- Acrylamide (29:1)

- UGB (Upper Gel Buffer) (5x)

For 900 mL of deionized H_2O add 75.69 g of Tris. Mix until the solute has dissolved. Adjust the PH to 6.8 and the volume to 1 L with deionized H_2O.

- LGB (Lower Gel Buffer) (4x)

For 900 mL of deionized H_2O add 181.65 g of Tris and 4 g of SDS. Mix until the solute has dissolved. Adjust the PH to 6.8 and the volume to 1 L with deionized H_2O.

- 10% APS (Ammonium Persulfate)

In 10 mL of deionized H_2O dissolve 1 g of APS. Note: Always use a new APS solution.

- 10% SDS (Sodium dodecilsulfate)

In 10 mL of deionized H_2O dissolve 1 g of SDS.

- Loading Gel Buffer (LB) (4x)

Tris Solution (PH 6.8) 1mM	2.5 mL (250 mM)
SDS	0.8 g (8%)
Glicerol	4 mL (40%)
Beta-Mercaptoetanol	2 mL (2%)
Bromophenol blue	1 mg (0.01%)

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

- Tris 1M (PH 6.8) solution

  For 150 mL of deionized H_2O add 30.3 g of Tris base. Adjust the PH to 6.8 and the final volume to 250 mL.

- Running buffer (10x)

Tris	30.3 g (250 mM)
Glycine	144.2 g (2.5 mM)
SDS	10 g (1%)

Dissolve in deionized H_2O and adjust the PH to 8.3 and the final volume to 1 L.

7.5 – Western-Blotting

Equipment:

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

Reagents and Solutions:

- Transfer buffer (1x)

Tris	3.03 g (25 mM)
Glycine	14.41 g (192 mM)

Mix until the solutes have dissolved. Adjust the PH to 8.3 with HCL and the final volume to 800 mL with deionized H_2O. Just before usage add 200 mL of methanol (20%).

7.6 – Immunoblotting

- 10x TBS (Tris Buffered Saline)

Tris	12.11 g (10 mM)
NaCl	87.66 g (150 mM)

Adjust the PH to 8.0 with HCL and the final volume to 1 L with deionized H_2O.

- 10x TBS-T (TBS-Tween 20)

Tris	12.11 g (10 mM)
NaCl	87.66 g (150 mM)
Twenn 20	5 mL (0.05%)

Adjust the PH to 8.0 with HCL and the final volume to 1 L with deionized H_2O.

- Ponceau S solution

Dissolve 0.1 g of Ponceau S (Sigma) in 100 mL 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

- Developer and fixer solution (Sigma)

- Membrane stripping solution

Tris-HCL (PH 6.7)	3.76 g (62.5 mM)
SDS	10 g (2%)
Beta-mercaptoethanol	3.5 mL (100 mM)

Dissolve Tris and SDS in deionized H_2O and adjust the PH to 6.7. Add the mercaptoethanol and adjust the final volume to 500 mL.

7.7 – Quantitative analysis

Equipment:

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

7.8 – Immunocytochemistry

Equipment:

- IX81 Motorized Inverted Microscope (Olympus Corporation, Hamburg, Germany) and analySIS 3.2 software (Olympus)
- ImageJ software
- LSM510 – Meta Confocal Microscope (Zeiss) and Zeiss LSM510 4.0 software

Reagents and Solutions:

- Coverslips (15 mm) (Thermo Scientific)
- 4% PFA (Sigma)

For 50 mL of deionized H_2O add 4 g of PFA. Mix until the solute has dissolved, at 58ºC. Adjust the PH to 6.9 and the final volume to 100 mL with PBS 2x. Filter and store at RT in the dark.

- Permeabilizating solution

0.2% Triton in 1x PBS.

- Blocking solution

3% of BSA (Bovine serum albumin, NZytech) in 1x PBS.

- Vectashield with DAPI
- Antibodies

	1st Antibodies	2nd Antibody
Acetylated α-Tubulin	Mouse anti-acetylated α-tubulin, clone 6-11B-1 Dilution 1:250 (Sigma)	Alexa Fluor 488 Dilution 1:300 (Life Technologies)
F-Actin	Alexa Fluor 568 Phalloidin Dilution 1:1000 (Life Technologies)	

7.9 – Statistical analysis

Equipment:

- GraphPad Prism version 4.0 software.