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NEUROINFLAMMATION IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE: A CENTRAL ROLE FOR PERIPHERAL MONOCYTES

Surname ANGIULLI Name FEDERICA

Registration number 848185

Tutor: Prof. CARLO FERRARESE

Co-tutor: Prof. LUCIO TREMOLIZZO

Supervisor: Dr. ELISA CONTI

Coordinator: PROF. ROSA MARIA MORESCO

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TABLE OF CONTENTS

ABSTRACT

Background and Aims: Neuroinflammation is a key event in Alzheimer's disease (AD) and is sustained by resident glial cells and blood derived monocytes attracted into the brain. Still, monocytes contribution to AD is controversial. Therefore, the aim of this study is to investigate monocytes recruitment in the AD brain and to understand their involvement in Aβ clearance. The contribution of CCR2 and TSPO receptors to the regulation of chemotaxis was assessed, toghether with that of TREM2 and its soluble form (sTREM2) to phagocytosis. Moreover, the disease-modifying potential of Donepezil, Co-ultraPEALut and anti-Aβ monoclonal antibodies (mAb) - in relation to their ability to influence these processes - was evaluated. Finally, the potential implication of neuroinflammation and the DBI/TSPO system in the agitation/aggression (A/A) cluster of Behavioral and Psychological Symptoms of Dementia (BPSD) was assessed.

Materials and Methods: Boyden chamber chemotaxis assays and fluorescence microscopy-based phagocytosis assays were performed on monocytic cell lines and monocytes/macrophages from AD patients and controls. Oligomeric Aβ42 was used as chemoattractant and phagocytic target; the assays were also performed upon stimulation with Donepezil, Co-ultraPEALut and anti-Aβ mAb (at pathologically low levels). Expression of CCR2, TSPO and TREM2 were investigated through Real-time PCR and Western Blot analysis; plasma levels of sTREM2 were measured by ELISA. DBI levels were assessed by ELISA in CSF and serum of A/A patients. TSPO expression in lymphomonocytes from A/A patients was determined by Real-time PCR and Western Blot. Migration of monocytes from A/A patients was quantified through Boyden chamber assay.

Results: Aβ42 promotes monocytes migration, but is not able to modulate CCR2 and TSPO expression. Monocytes from AD patients have reduced TREM2 expression, suggestive of limited phagocytic activity. Donepezil inhibits Aβ-induced migration, and impacts the phagocytic activity of cell lines and human macrophages from healthy controls; however, it fails to show any effect in macrophages from AD patients. Co-ultraPEALut prevents Aβ-induced chemotaxis and increases TREM2 expression in macrophages, probably recovering their phagocytic competence. Anti-Aβ mAb decrease Aβ-induced migration, but are not able to increase phagocytosis. DBI levels and TSPO expression do not increase in A/A patients, and monocytes from A/A patients do not show any difference in terms of chemotactic activity compared to their counterparts.

Discussion: Taken together, these findings suggest an involvement of Aβ42 in the chemotaxis of monocytes in AD and a reduced phagocytic activity charachterizing macrophages from AD patients. The results fail to completely elucidate the mechanisms underlying Aβ-induced migration, even

though they clearly point towards an involvement of TSPO in the process. Donepezil and Co-ultraPEALut emerge as useful therapeutic agents with the potential to counteract neuroinflammation by modulating chemotaxis and phagocytosis, despite treatment response in AD patients requiring additional investigations. On the other hand, an increase in specific anti-Aβ mAb in the brain of AD patient is required to deliver a protective effect in terms of plaque clearance, despite pathologically low intrathecal levels being already sufficient to interfere with monocyte recruitment. Finally, data suggest that the DBI/TSPO system may not be involved in A/A pathogenesis.

Conclusion: In the future we propose to elaborate on the modulation of receptors involved in chemotaxis and to complete the characterization of the phagocytic phenotype of peripherally-derived macrophages. Future studies will be also aimed at validating the therapeutic use of the selected disease-modifying compounds. Finally, more experiments will be necessary to understand if neuroinflammation could play a role in the pathogenesis of other BPSD clusters.

Chapter 1
Alzheimer's Disease

1.1 Alzheimer's disease overview

Alzheimer's disease (AD) is an age-related neurodegenerative disorder defined by progressive cognitive deterioration, affecting both memory and other aspects of cognitive functioning. It is recognized as the most common cause of dementia, accounting for 60% to 80% of all cases, with a prevalence of around 10% after 65 years of age - increasing to about 40% in people aged 85 years or older [1].

From a biological point of view, the disease is characterized by the accumulation of misfolded proteins in the aging brain, namely extracellular amyloid plaques - deposits of β amyloid (Aβ) peptides - and intracellular neurofibrillary tangles (NFTs) of hyperphosporylated tau protein [2]. The downstream consequences of this pathological process include neurodegeneration with synaptic and neuronal loss leading to brain atrophy and cortical thinning [2]. Even though alterations have been found in the frontal, parietal and occipital lobes [3], one of the most compromised regions is the hippocampus, which is fundamental for mnemonic processes [4]. Indeed, episodic and spatial memory are damaged at first, followed by executive functions in later stages of the disease [5,6].

1.2 Pathological subtypes

Two different pathological subtypes of AD have been identified: the familial or early-onset AD (EOAD) accounts for approximately 1% to 6% of all cases, occurs in young individuals (normally under 65 years of age) and is associated with autosomal dominant missense mutations in different genes [7]; on the other hand, the sporadic or late-onset form (LOAD) accounts for 95% of all cases and develops as a result of environmental and genetic factors [8,9].

- ***Early-onset AD***

The first missense mutation responsible for EOAD has been identified in the amyloid precursor protein (APP) gene on chromosome 21 [10]. However, additional mutations can occur in homologous presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes - on chromosome 14 and 1 respectively - encoding for the

catalytic subunit of γ-secretase [11,12]. These mutations lead to an impairment in the amyloidogenic pathway, inducing an increased accumulation of the Aβ peptides [13].

- ***Late-onset AD***

LOAD has a complex etiology, probably developing as a result of multiple risk factors, including susceptibility genes and environmental aspects [14].

The strongest genetic risk factor for LOAD is represented by the ApoE gene, located on chromosome 19q13 and encoding for a 299-amino-acids-long glycoprotein named apolipoprotein E. The protein is normally produced by astrocytes and microglia, but can also be expressed by neurons following stress or cellular damage; it is a component of lipoprotein particles, binding to cholesterol, triglycerides and phospholipids [15,16]. The ApoE gene has displays three different alleles (ε2, ε3 and ε4), conferring conformational differences to the ApoE protein and thereby influencing its ability to bind Aβ and promote its clearance [17,18]. In particular, ApoEε4 carriers show impaired Aβ clearance and therefore an increased deposition of amyloid fibrils; in addition, ApoEε4 fragments tend to accumulate in neurofibrillary tangles and amyloid plaques, disrupt cytoskeletal structure, and impair mitochondrial function [19,20]. As a consequence, individuals homozygous or heterozygous for ApoEε4 present a higher risk of developing AD compared to non-carriers [21].

Thanks to genome-wide association studies (GWASs), many other genes have been identified as potential risk factors for AD - including CD33, TREM2, ABCA7, CLU, CR1, CD2AP, MS4A and MEF2C - the corresponding proteins participating in a variety of cellular processes, such as oxidative balance, protein metabolism, cholesterol metabolism and synaptic function [22].

Beyond genetics, the major risk factor for LOAD is age, with a sharp increase in incidence after 60 years [23]. In addition, strong environmental and metabolic predisposing factor could facilitate the onset of LOAD - including hypercholesterolemia, obesity, type 2 diabetes mellitus, hypertension and hyperhomocysteinemia. Indeed, AD patients usually display high plasma levels of cholesterol, able to induce APP cleavage by means of γ-secretases; by contrast, the inhibition of cholesterol biosynthesis has been shown to increase α-secretase activity, favoring neuroprotection [24,25]. A different connection has been found between LOAD and obesity: this food disorder is characterized by high circulating levels of lipids and glucose, a condition leading to the development of chronic inflammation and oxidative stress - contributing to neuroinflammation and neurodegeneration [26,27]. Likewise, an increased risk of LOAD has been linked to diabetes, by means of several suggested mechanisms - including, insulin resistance and deficiency, toxicity due to hyperglycemia, cerebrovascular damage and inflammation [28]. As for

hyperhomocysteinemia and hypertension, these conditions contribute to LOAD by giving rise to blood-brain barrier (BBB) dysfunctions, brain atrophy and NFTs formation [29].

1.3 Etiopathogenesis

AD etiopathogenesis is far from being completely elucidated, and the event responsible for triggering the neurodegenerative process is still unknown. To date, several theories have been formulated, however, given the complexity of the disease, it is more likely to hypothesize a multifactorial etiology - a combination of different factors rather than one single cause.

- ***The Amyloid Cascade Hypothesis***

The amyloid cascade hypothesis traces AD pathogenesis back to a set of anomalies in both the productive and secretive pathway of the amyloid precursor protein (APP), a transmembrane glycoprotein expressed in neuronal tissues [30].

In the brain under physiological conditions, APP undergoes a non-amyloidogenic processing by means of α-secretase (ADAM 9, ADAM10 or ADAM17 [31]) and γ-secretase [32]- a heteromeric complex consisting of four essential subunits: presenilin-1 (PSEN-1) or presenilin-2 (PSEN-2), nicastrin (NCSTN), anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN- 2) [33]. The first cleavage results in the secretion of the soluble extracellular domain of APP (sAPPα), endowed with neurotrophic and neuroprotective functions, as well as the C83 C-terminal fragment; whereas the second cut generates two additional fractions, the p3-peptide and a small APP intracellular domain (AICD) portion [34]. However, APP may undergo a second - amyloidogenic - processing. In this case, the first cut is performed by the β-secretase BACE1 (β-site APP-cleaving enzyme 1) and generates both a soluble APPβ (sAPPβ) peptide and a membrane-bound carboxyl terminal fragment (C99); subsequently, C99 is cleaved by the γ-secretase to generate the AICD portion and the amyloid β peptide (Aβ) [34]. Of note, the γ-secretase processing is not precise, leading to the release of various Aβ peptides differing in length (most commonly Aβ40 and Aβ42, ending at amino acid 40 and 42 respectively) [35].

Aβ is poorly soluble, and the release into the extracellular space prompts its aggregation into progressively larger polymers and accumulation in plaques [36]. Of note, AD senile plaques are composed primarily of Aβ42, far more toxic and more prone to aggregation than Aβ40 [37]. The proposed amyloid cascade hypothesis assumes that AD is caused by an imbalance between Aβ production and clearance, resulting in increased Aβ burden in the brain and plaques formation, culminating in neuronal damage

and death [30]. Overproduction of Aβ is critical to the pathogenesis of some cases of EOAD [38]; nevertheless, Aβ dysregulation in the far more common LOAD seems to be the result of ageing and the impairment of Aβ degradation [39] and/or clearance [40].

However, Aβ aggregation in fibrils and deposition in plaques does not appear to be the only pathogenic trigger for AD. Soluble oligomers represent the most toxic species of Aβ [41] and can contribute to AD progression and cognitive decline [42]. Functional disruption of NMDA receptors [43], increased plasma membrane permeabilization [44], mitochondrial dysfunction and oxidative stress [45], inflammation [46] and synaptic disfunction [47] are just a few mechanisms. Of note, these detrimental effects seem to be related to Aβ oligomers size and critical concentration [48].

- ***Tau hyperphosphorylation***

Hyperphosphorylation and aggregation of protein tau represents another pathological hallmark of AD and putative pathogenic mechanism for the disease. Tau is a microtubule-associated protein normally present in neurons, where is responsible for axonal microtubules stabilization [49]. Alternative splicing of the microtubule-associated protein tau (MAPT) gene give rise to six tau isoforms - differing for the number of exons constituting their mRNAs - all present in the adult human brain [50].

In AD, tau can become hyperphosphorylated as a result of a dysfunctional phosphorylation process: it loses the ability to interact with microtubules and starts to aggregate in intracellular neurofibrillary tangles (NFTs), with subsequent impairment of the axonal transport [51]. As it happens for Aβ, soluble monomers are recruited to the already formed NFTs throughout disease progression [52] and the oligomers seem to be the most critical mediators of tau toxic effects [53]. The existence of a link between Aβ toxicity and tau pathology has been established, albeit the elusive underlying mechanisms [54]: some evidences suggest that tau oligomerization could be a consequence of Aβ accumulation [55]; others, on the other hand, propose tau as a mediator of Aβ toxicity. In support of this last hypothesis, it has been demonstrated that tau deficiency is able to attenuate Aβ-induced neuronal damage and death [56].

- ***Cholinergic hypothesis***

The cholinergic hypothesis focuses the attention on the role of acetylcholine in the onset of the disease. The dysfunction of cholinergic neurons in the basal forebrain and hippocampus - critical regions for attention, learning and memory - considerably contributes to the cognitive decline in AD [57]. Deficits in the cholinergic transmission are associated to alterations of choline uptake and acetylcholine release,

alteration of nicotinic and muscarinic receptors expression, and down-regulation of specific enzymes - such as acetyltransferase and acetylcholinesterase [58,59].

In light of the interplay existing between the two systems, cholinergic dysfunctions can affect the glutamatergic transmission: as a matter of fact, acetylcholine is thought to be protective against Aβ-induced NMDA glutamate receptors hyperactivation, crucial for neurodegenerative processes in AD [60,61].

Of note, despite the important role of the impaired cholinergic (and glutamatergic) neurotransmission in the pathology, it can unlikely be considered as a definitive cause on its own [62].

Beyond amyloid plaques deposition, tau hyperphosphorylation and cholinergic dysfunction, it is now recognized that other pathological processes - strictly connected to each other - can contribute to AD pathogenesis just as much as its pathological hallmarks. Neuroinflammation, neuronal and synaptic degeneration, cerebral homeostasis disruption, metabolic disorders, vascular dysfunction and oxidative stress are just a few examples [63-69].

1.4 Alzheimer's disease continuum

It is possible to identify three different stages during the progression of AD: preclinical phase, mild cognitive impairment (MCI) and AD dementia.

The preclinical stage starts early in the lifetime and, even though brain changes have already started to appear, it is totally asymptomatic since the brain is still able to compensate for them [70]. Slowly, cognitive decline starts to interfere with everyday activities, compromising functional autonomy and making the impairment become visible [71].

The MCI stage is characterized by the evidence of Alzheimer's brain changes plus reduced cognitive abilities that - however - do not interfere with the ability to independently carry out everyday activities. MCI can be categorized into “amnestic” - manifesting with reduced memory performances - and “non amnestic” - characterized by a decline in attention, language and executive functions, which can be more or less evident [72]. Of note, MCI does not always convert to dementia and can conversely revert to normal cognition [73].

As Alzheimer's progresses to dementia, memory, thinking or behavioral symptoms starts to appear; those manifestations change with time, following the pattern of damage striking different areas of the brain, and advance from mild to moderate to severe [74].

1.5 Behavioral and Psychological Symptoms of Dementia

The term Behavioral and Psychological Symptoms of Dementia (BPSD) was introduced to describe a wide variety of disturbances usually manifesting during the course of AD pathology [75]. These symptoms include anxiety, agitation, apathy, depression, psychosis, aggression, euphoria, disinhibition, hallucination, elation, delusion, misidentification, dysphoria, irritability, wandering, aberrant motor behavior, eating disorders and sleep disturbances [76], and can be categorized into clusters based on the symptom profile: affective symptoms (dysphoria, anxiety and apathy), psychosis (delusions and hallucinations), hyperactivity (irritability, aggression, disinhibition and aberrant motor behavior) and euphoria [77].

BPSD have a 90% prevalence in dementia, and almost all patients experience at least one of them during the course of the disease with apathy and agitation showing the highest incidence [78,79]. They can be either episodic or persistent over long periods of time, and appear at any time during the progression of the disease - MCI stage included [80] - despite having higher prevalence rates in the middle stages of dementia [81].

The main clinical instrument used to measure BPSD is the Neuro Psychiatric Inventory (NPI), allowing the assessment of 12 different disturbances (delusions, hallucinations, agitation, dysphoria, anxiety, apathy, irritability, euphoria, disinhibition, aberrant motor behavior, night-time behavior disturbances, and appetite and eating abnormalities). The test is administered to caregivers to determine the frequency and severity of symptoms and to evaluate the distress they experience while assisting the patients [82].

A complex interaction of biological, psychosocial/psychological and environmental factors seems to give its contribution to the development of BPSD, but the exact causes are far from being completely clarified. Moreover, a definite pharmacological treatment has not yet been identified. The management of BPSD is highly individualized but generally non-pharmacological treatment should be attempted first, prior to pharmacological intervention. Non-pharmacological interventions include psychosocial/psychological counseling, interpersonal management and environmental management, while the most employed symptomatic therapies are antidepressants, mood stabilizers, cognitive enhancers and antipsychotics [83].

1.6 Diagnosis

The definitive diagnosis of AD is based on post-mortem histopathological detection of characteristic pathologic brain lesions - Aβ plaques and neurofibrillary tangles - similar to those described by

Alzheimer himself in 1907 [84,85]. Additionally, the routine medical practice - based mostly on clinical and neuropsychological assessment in vivo - makes it possible to diagnose merely probable or possible AD [74]. Attempts have been made in the last few years to get to an in vivo neuropathological diagnosis of the disease, independent from clinical symptoms [86]; however, the latest recommendation is to opt for a comprehensive approach, encompassing both clinical assessment and in vivo neuropathological evaluation [87]. In the current situation, it is therefore possible to diagnose this disease uniquely in its symptomatic stage, when significant brain damage has already occurred and cognitive deficits have become unmistakable.

- ***Neuropsychological tests***

Montreal Cognitive Assessment (MoCA) [88], Alzheimer's Disease Assessment Scale, Cognitive subscale (ADAS-Cog) [89], Disability Assessment of Dementia (DAD) [90] and Mini-Mental State Examination (MMSE) [91] are just a few of the tests available for AD neuropsychological assessment. MMSE, in particular, is the most widely used. The MMSE total score - ranging from 0 to 30 - correlates with disease progression: it is therefore possible to identify mild (score 21-26), moderate (score 15-20), moderately severe (score 10-14) and severe (score <10) dementia [92].

- ***Imaging***

Neuroimaging techniques for the diagnosis of probable AD include magnetic resonance imaging (MRI) and Positron Emission Tomography (PET) [93].

MRI provides high-resolution cerebral pictures, allowing the evaluation of functional and structural abnormalities. In particular, AD patients show atrophy of the temporal lobe, ventricular enlargement and reduced brain volume [94].

PET, on the other hand, is a functional technique using radioactive tracers specific for various imaging purposes. In particular, FDG-PET - making use of 18-fluorodeoxyglucose (18F-FDG) tracer - is employed for the in vivo assessment of brain metabolism variations (often preceding structural brain changes). In AD patients, this technique can reveal a lower glucose consumption compared to healthy controls [95]. Differently, the Pittsburgh compound B (PiB) allows the in vivo visualization of amyloid fibrils with high sensitivity. Of note, AD patients show an increased tracer uptake compared to controls, and present a pattern of amyloid plaque deposition corresponding to the one observed in post-mortem examinations [96]. [C11]PK11195, instead, is a high selective tracer for activated microglia - binding to

the TSPO receptor on their plasma membrane. As a matter of fact, TSPO levels experience a dramatic increase in AD in response to neuroinflammation as a result of glial activation (and peripheral monocytes recruitment) [97]. Finally, PET might be used to track tau protein and neurofibrillary tangles [98].

Despite their usefulness, brain imaging techniques are really expensive, require trained staff to perform the examination and are not applicable to large groups of patients. Therefore, clinical evaluation is more commonly accompanied by biological fluids-based biomarkers analysis.

- ***Biological fluids-based biomarkers***

Universally, the term *biomarker* refers to whatever feature that can be objectively measured and that is able to define the status of a biological entity at a given time [99]. The ideal biomarker for Alzheimer's disease should closely reflect the succession of pathological events and prove potentially useful for a wide variety of applications. First and foremost, biomarkers are expected to be helpful for the early and differential diagnosis of AD. They should be able to reveal or suggest the existence of the fundamental alterations of the AD brain even during the preclinical asymptomatic phases of the disease: a better understanding of the biomarker signature preceding the clinical manifestation would enable the differentiation between individuals at risk for AD and elderly people experiencing the normal aging process. At the same time, biomarkers should be able to correlate with disease intensification – with the purpose of discerning between the different stages of AD pathology and predicting conversion and progression to further cognitive decline – and to discriminate AD from other dementias [100].

Given its intimate contact with the central nervous system (CNS), the cerebrospinal fluid (CSF) represents a reasonable source for AD biomarkers. As a matter of fact, the CSF reflects the status of the CNS and, therefore, pathological changes in the brain – determining alterations in the level of various substances – are able to affect its composition accordingly [101]. Both research and clinical practice have largely benefitted from biomarker discovery studies in the CSF over the years, so much that the measurement of AD specific biomarkers (Aβ, Tau and pTau) in the CSF has been included in the criteria for the in vivo diagnosis of this disease [102]. However, the high invasiveness of CSF collection by lumbar puncture - together with the risks and costs associated with the procedure – has extensively limited its application in standard clinical routine, given the reluctance of patients to undergo invasive and expensive testing repeatedly.

The limitations of CSF have prompted a switch of attention towards more accessible biological sources for biomarker research. The current focus is primarily on blood, whose collection is a routine procedure

undeniably less invasive, inexpensive, replicable at regular intervals and easy to implement in large populations, compared to lumbar puncture. As a consequence, blood-based biomarkers appear to be easily implementable in clinical practice to monitor disease progression or treatment efficacy over time [103]. However, the analysis of blood biomarkers comes with an array of detection challenges, imputable to the nature of the sample and to the complexity of AD pathology, and it could be often difficult to correlate changes in the CNS with those observed in blood [103-106].

1.7 Pharmacological therapy

The therapies currently administered to patients with advanced disease are just symptomatic, and thus unable to block the pathophysiological events leading to full-blown AD. Differently, it would be ideal to make use of the so-called "disease-modifying therapies" - treatments able to able to act on the cause and evolution of the disease by interfering with its pathogenesis and counteract or delay the appearance of symptoms [107].

- ***Acetylcholinesterase inhibitors***

First class of FDA approved drugs for mild to moderate AD treatment are the acetylcholinesterase inhibitors (AChEI), such as Donepezil, Rivastigmine and Galantamine. AChEI exert their pharmacological action by inhibiting acetylcholine (ACh) degrading enzyme (acetylcholinesterase), increasing ACh levels in the brain and thereby restoring cholinergic transmission - impaired in AD due to Aβ accumulation – with positive effects on cognition [108]. Interestingly, AChEI have also demonstrate the ability to modulate the inflammatory response (disease modifying action) [109]. These compounds are well tolerated, regardless of their dose-dependent side effects [110].

- ***Memantine***

Memantine is an NMDA antagonists approved by the FDA for the treatment of moderate to severe AD [111]. This molecule prevents glutamate neurotoxicity - induced by the overstimulation of its membrane receptors and the excessive subsequent Ca^{2+} influx through the associated ion channels - without interfering with its physiological actions, necessary for learning and memory. Memantine is thus well tolerated [112]. Of note, because of their different mechanism of action, Memantine can be can be prescribed in combination with AChE inhibitor Donepezil [113].

- ***Immunotherapy***

On 7 June 2021, Aducanumab became the first ever disease-modifying medication approved by the FDA to treat patients with MCI or mild dementia stage of AD [114]. Of note, it is also the first therapeutic agent approved for AD since 2003. Being a human monoclonal antibody selectively interacting with Aβ aggregates - either soluble oligomers or insoluble fibrils [115] - Aducanumab exerts its mechanism of action by crossing the BBB and reducing the Aβ deposits in the brain: in fact, Aβ plaques measured by amyloid PET in the AD patients enrolled in phase 3 studies resulted markedly decreased by Aducanumab in a dose- and time-dependent manner [116]. Moreover, a reduction in pTau in the CSF and NFTs measured by tau PET were also observed in a small subset of patients [117]. More importantly, the EMERGE trial met the primary endpoint, showing a reduction in clinical decline in patients treated with high-dose Aducanumab [117,118]. Aducanumab treatment is associated with the occurrence of Amyloid-Related Imaging Abnormalities (ARIA) side events, usually manageable (headache) ore even asymptomatic [116,117].

1.8 Non-Pharmacological Strategies

Even though pharmacological treatments are still the gold standard for the treatment of AD, several studies have focused on preventive drug-free strategies.

Physical activity, mental exercise and socialization can reduce the risk of AD dementia - and may enhance overall cognitive functions [119] - by promoting brain plasticity [120,121]. Moreover, physical exercise may lower the susceptibility to cardiovascular disease and diabetes, common risk factors for AD [122].

Early findings from few small studies have shown the potential effect of dietary practices on the management of AD. More specifically, adherence to the Mediterranean diet seem to be associated with improved cognitive functioning and reduced amyloidosis and tauopathy [123]. At the same time, it may exert a protective role over oxidative stress, inflammation and cardiovascular risk factors [124].

Currently the attention has converged on the potential neuroprotective role of natural compounds, able to attenuate or suppress inflammation and oxidative stress.

- ***Co-ultraPEALut***

Co-ultraPEALut is a co-ultramicronized compound constituted by the association of N-palmitoylethanolamine (PEA) and the antioxidant flavonoid luteolin, showing anti-inflammatory and

antioxidant properties [125]. Co-ultraPEALut based products (e.g. Glialia®) are already licensed for use in humans as nutraceuticals or food for medical purposes. Published findings on experimental models of AD demonstrated the beneficial effect of Co-ultraPEALut treatment on both cognition and a range of neuropathological features of AD, including Aβ-induced neuroinflammation and neuronal toxicity [126,127]. Furthermore, recent studies have emphasized the ability of this compound to counteract cellular and molecular modifications already in the prodromal stage of AD [128]. Clinical data concerning the putative beneficial effect of Co-ultraPEALut in AD patients are lacking. However, in line with the aforementioned findings on preclinical models, a single case-report study revealed significant improvement in the neuropsychological performances of an MCI patient treated with high-dose Co-ultraPEALut [129].

Chapter 2
Neuroinflammation

2.1 Neueroinflammation in the pathogenesis of Alzheimer's disease

The term *neuroinflammation* refers to the broad range of inflammatory responses originated in the CNS secondary to insults, injury, or disease. Neuroinflammation has been described in AD since the first characterization of the disorder [84], and is now well recognized as a central feature in its development, contributing to the pathogenesis just as much as the pathological hallmarks [130]. The pathological accumulation of Aβ and NFT in the brain is considered the principal trigger for neuroinflammatory responses in AD, inducing the activation of resident glial cells [131].

- ***Microglia***

Microglia are the resident innate immune cells of the CNS. They are ubiquitously distributed in the brain and act as first line of defense, playing a fundamental role in its surveillance [132]. In AD, microglia are able to identify and bind Aβ - through a range of different receptors - and become activated [133]. When activated, microglia go through morphological changes, localize in the vicinity of senile plaques and start releasing inflammatory mediators [63]. It should be noted that microglial activation is a complex process, producing multiple phenotypes according to the evolution of the inflammatory response, likely to have either beneficial or detrimental roles and effects. Classical (M1) form of microglial activation is characterized by elevated production of pro-inflammatory cytokines - including tumor necrosis factor-alpha (TNF-α), interleukins IL-1β, IL-6, IL-12, IL-18, interferon gamma (IFN-γ), chemokines like the monocyte chemotactic protein 1 (MCP-1) and neurotoxic agents - and is accompanied by impaired phagocytic capacity. On the other hand, alternative (M2) activation phenotype is characterized by the release of several anti-inflammatory cytokines - interleukins IL-4, IL-10, IL-13 and transforming growth factor-beta (TGF-β) - tissue repair promotion and enhanced phagocytic ability [134,135]. Throughout AD progression, microglia switch from M2 to M1 activation phenotype. The immediate activation of microglia in AD leads to a defensive response (acute inflammatory response) aimed at promoting Aβ clearance, thereby reducing its accumulation [136]. However, the long-standing exposure to Aβ - due to

its increased production and accumulation over time - and inflammatory mediators (result of the pro-inflammatory environment) induce a functional impairment in the microglia, whose phagocytic capacity becomes largely insufficient [137]. The failure of microglial phagocytosis increases the amyloid burden in the brain and facilitates direct Aβ-induced neurotoxicity [138]. On its turn, neuronal damage contributes to the release of further pro-inflammatory cytokines by activated microglia. This long-standing and self-perpetuating (chronic) neuroinflammatory response is often detrimental, resulting in neurodegeneration and brain function impairment [139].

- ***Astrocytes***

Astrocytes are the most abundant glial cells within the brain, part of the resident innate immune system of the CNS and active players in the neuroinflammatory response [140]. Astrocytes constitute a structural part of the BBB, with their processes making extensive contacts with - and providing almost complete coverage of - brain microvessels [141]. In AD, astrocytes are intimately associated with Aβ deposits, with their processes surrounding and even penetrating into the plaques to isolate the neighboring healthy tissue. Astrocytes accumulating around senile plaques are markedly hypertrophic and reactive, showing a striking upregulation of the glial fibrillary acidic protein (GFAP) [142,143]. Reactive astrocytes are involved in Aβ clearance across the BBB through the Aquaporin 4 (AQP4) [144] and degradation [145]. Upon exposure to Aβ, reactive astrocytes release pro-inflammatory cytokines (mainly IFN-γ, IL-1β, IL6, TNFα and TGFβ) and up-regulate the production of reactive oxygen species (ROS) and nitric oxide (NO) [146]; those mediators act either in an autocrine and a paracrine way, modulating microglial activation [147] and self-perpetuating reactive gliosis. The persistent (chronic) activation of astrocytes results in atrophy and degeneration, decreased endfeet coverage of cerebral microvessels and altered AQP4 perivascular localization [148]; as a consequence, Aβ clearance through the BBB is compromised, with subsequent increase of the Aβ burden in the brain [149].

- ***Blood-brain barrier***

Pro-inflammatory mediators released by chronically activated glial cells in the AD brain could compromise the integrity and permeability of the BBB [150]. Under physiological conditions, the BBB completely separates the CNS from the peripheral immune system [151]; however, pro-inflammatory cytokines, chemokines, NO, ROS and metalloproteinases (MMPs) are able to increase BBB permeability, ultimately leading to the withdrawal of the immune privilege of the brain. In particular,

pro-inflammatory cytokines and oxidative stress modulate BBB permeability by acting on the tight junctions in the cerebrovascular endothelial cells [152,153], while MMPs digest the endothelial basal lamina necessary for BBB integrity [154]. Major contributor to this process is also cerebral amyloid angiopathy (CAA), a condition characterized by the accumulation of Aβ in the cerebrovasculature: amyloid-laden vessels - often associated with degenerated smooth muscle cells, pericytes and endothelial cells - show reduced integrity, with implications for BBB permeability [155].

2.2 Involvement of peripheral immune system in neuroinflammatory response in Alzheimer's disease

It is now widely accepted that the peripheral immune system is involved in the pathogenesis and progression of AD. As aforementioned, microglial and astrocytic activation leads to the release of soluble inflammatory mediators that, despite being released locally, are able to cross the BBB, diffuse into the bloodstream and migrate to the periphery, thereby recruiting peripheral immune cells [156]. At the same time, the CNS is particularly responsive to peripheral immune system activation: pro-inflammatory cytokines - released by peripheral cells in response to pathogens and injury - are transported by the blood-flow towards the BBB, where they cross into the brain parenchyma - either passively, through leaky areas of the barrier, or by active transport systems (receptors on epithelial cells) [157] - or, alternatively, stimulate the afferent vagus nerve to induce signaling within the brain [158]. This increased inflammatory trigger is able to act upon resident immune cells, promoting their activation and shift towards a pro-inflammatory phenotype [159].

- ***Cytokines and chemokines***

Cytokines released by immunocompetent cells - whether they be peripheral immune cells or resident glial cells - promote the bidirectional cross-talk between the AD brain and the periphery. IL-6, IL-1β and TNF-α can be considered among the principal mediators of the pro-inflammatory response in AD. In the CNS, these inflammatory mediators are released as a result of glial exposure to Aβ [146,160], while in the periphery they are commonly produced by monocytes/macrophages during the acute phase of the immune response [161]. IL-10, IL-4 and TGF-β may be regarded as the anti-inflammatory cytokines putatively able to limit the pro-inflammatory activation in AD. Their main activities concern the suppression of macrophages activation and the reduction of pro-inflammatory cytokines synthesis [162-165].

Chemokines are chemotactic cytokines essential for activation and migration of specific subsets of leukocytes in order to elicit a targeted and specialized immune response. In AD, the development of neuroinflammation is accompanied by a generalized upregulation of plaque-associated chemokines and chemokine receptors, leading to peripheral monocytes recruitment and glial cell activation [166,167]. Monocyte chemotactic protein 1 (MCP-1) - also known as CC chemokine ligand 2 (CCL2) - was the first chemokine associated with AD, and plays a pivotal role in the recruitment and accumulation of immune cells at the level of senile plaques. Inflammatory responses mediated by MCP-1 are linked to Aβ pathology, as Aβ-induced upregulation of MCP-1 has been demonstrated in microglia, astrocytes and human monocytes [168,169]. It is plausible that plaque-associated MCP1 production is related to an initial attempt of glial cells to eliminate Aβ deposits by means of resident and peripheral phagocytes recruitment [170,171]; however, its overexpression in late stages of AD may induce inflammatory states with detrimental effects on the brain [172].

- ***Monocytes/Macrophages***

Circulating monocytes are predominantly recruited in the AD brain in a CCR2/CCL2-dependent manner. CCR2 is a monocytic surface receptor for CCL2 (also known as MCP-1), one of the most effective chemotactic factors for monocytes, upregulated near Aβ deposits as a result of the release from plaque-associated microglia [166]. Noteworthy, the process can be potentiated by the concurrent intervention of soluble Aβ, proven to be a powerful chemotactic stimulus both in vitro blood-brain barrier models and in AD transgenic mice [156,173]. In addition, the translocator protein 18 kDa (TSPO) may give an additional contribution: indeed, previous ex vivo human studies have demonstrated both the presence of functional TSPO receptors on monocytes' surface [174] and the ability of various TSPO ligands to influence their chemotaxis [175].

The contribution of circulating monocytes to AD pathogenesis is still controversial. In vivo studies using mouse models of AD showed that the recruited monocytes are able to cross the BBB in the areas of greater permeability, reach the perivascular space and, from there, infiltrate the brain parenchyma, associating with regions of increased Aβ deposition [176]. Upon recognition of Aβ peptides, infiltrating monocytes appear to differentiate into macrophages and to contribute to the clearance of Aβ from both the brain vasculature and parenchyma, thereby restricting Aβ plaques and reducing the degree of CAA [173,177]. On the other hand, monocytes isolated from AD patients exhibit reduced differentiation into macrophages - together with decreased phagocytosis and lysosomal degradation of Aβ - compared to monocytes from age-matched healthy controls [178].

- ***Lymphocytes***

The role of the adaptive immune system in AD is far from being completely elucidated, however ablation of T and B lymphocytes results in significant acceleration of amyloid pathogenesis and worsening of neuroinflammation [179]. A number of reports have confirmed an increase of CD4+ and CD8+ T cells, in brain, blood and CSF of AD patients [180,181]. In addition, the number of B cells was found significantly increased in the blood of AD patients compared to healthy controls (given their role in antibodies' secretion, direct tissue infiltration is not necessarily required) [182]. T cells can interact with microglia and modulate their phagocytic and secretory phenotype, either by infiltrating the brain through the damaged BBB or exerting their function from the periphery (via cytokines' secretion) [183]. On the other hand, B cells can be induced by Th2 modulation to produce immunoglobulins - and, among them, anti-Aβ antibodies. Anti-Aβ antibodies have proved able to promote Aβ clearance - thereby reducing its deposition in plaques - via different mechanisms: they can bind Aβ in the brain and favor its degradation by microglial cells [184] or facilitate its efflux through the BBB [185]; alternatively, they can sequester Aβ in the peripheral blood, lowering its free level, and induce its release from the brain (peripheral sink effect) [186]. Furthermore, naturally occurring anti-Aβ auto-antibodies (NAb) may have a role in preventing Aβ toxicity and aggregation [109,187]. However, studies aimed at quantifying the amount of those anti-Aβ NAb in AD patients point towards a generalized reduction in both CSF [188] and periphery [189].

2.3 The cholinergic anti-inflammatory pathway

The CNS is able to monitor and regulate inflammatory responses in real time by means of the cholinergic anti-inflammatory pathway (CAIP) [190]. The so-called *inflammatory reflex* is triggered when peripheral inflammatory stimuli signal the nucleus tractus solitarius (NTS) by interacting with their receptors on the vagus nerve afferent fibers; once processed in the brain, the outbound signals are transmitted to the spleen via efferent vagal nerve and splenic nerve. Upon activation, the splenic nerve releases norepinephrine (NE), which interacts with β2-adrenergic receptors expressed on the choline acetyltransferase (ChAT)-positive T cells in the spleen, increasing acetylcholine (ACh) synthesis and release. Finally, the binding of ACh to α7 nicotinic acetylcholine receptors (α7nAChRs) expressed on monocytes/macrophages inhibits pro-inflammatory cytokine synthesis and release, resulting in alleviated systemic inflammation [191].

2.3.1 The α7 nicotinic acetylcholine receptor

Nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated pentameric ion channels, located on the cell surface and activated by the neurotransmitter acetylcholine. The α7 nicotinic acetylcholine receptor (α7nAChR) is a special subtype of nAChR, encoded by the CHRNA7 gene located on chromosome 15 and consisting of five homologous α7 subunits [192]. Although nicotinic receptors are originally described as sodium channels, the α7nAChR also presents a high permeability for calcium [193]. Binding of agonists produces conformational changes in the receptor that open its central pore allowing both calcium and sodium influxes, initiating cations-dependent signaling cascades [194]. Principal agonists of α7nAChR are acetylcholine and nicotine, while high affinity selective antagonists are α-bungarotoxin and methyllicaconitine (MLA) [195,196].

In the CNS the α7nAChR is present in neurons, both in the presynaptic and postsynaptic membrane. Activation of α7nAChR in the presynaptic membrane leads to the release of multiple types of neurotransmitters - including GABA, glutamate, acetylcholine and dopamine; at a postsynaptic level, the receptor plays an important role in synaptic plasticity, contributing to learning, memory and other cognitive functions [197]. In addition, such nicotinic receptors have been shown to be expressed by astrocytes [198] and microglia [199], where they mediate anti-inflammatory effects: in particular, activation of the α7nAChR might lead to a shift in microglia phenotype from proinflammatory to neuroprotective, similarly to what has been described for peripheral monocytes [200].

Outside the brain, the α7nAChR is expressed on several cells of the immune system, including monocytes/macrophages and lymphocytes [201]. As aforementioned, activation of the α7 receptor on monocytes/macrophages leads to the attenuation of pro-inflammatory cytokine release, playing a crucial role in the CAIP [191]. Furthermore, the presence of the α7nAChR has been reported in T and B lymphocytes [202,203], where its expression seems to be upregulated upon immune activation [204,205].

2.3.2 Role of the α7nAChR in Alzheimer's disease

An intra-subunit allosteric binding pocket within its transmembrane domain allows the α7nAChR to interact with Aβ, down to picomolar concentrations. The receptor exhibits increased binding affinity for Aβ42 compared to Aβ40, as well as for oligomeric Aβ42 compared to its fibrillar or monomeric form [206], and represents one of the most important receptors mediating Aβ phagocytosis: the binding to

oligomeric Aβ42 leads to the internalization of the complex through endocytosis and to its accumulation within the lysosomal compartment [207].

Under normal physiologic conditions, the interaction between Aβ42 at low (picomolar) concentrations and the α7nAChR on neuronal cells results in the activation of signal transduction cascades associated with neuroprotection and synaptic plasticity [208]. However, as AD progresses, chronic exposure to Aβ42 at nanomolar concentrations leads to α7nAChR inactivation - putatively through a desensitization mechanism - resulting in dysregulation of signal transduction mechanisms involved in normal receptor functioning [209]. Moreover, the interaction produced under those specific conditions may enhance receptor-peptide complex internalization, leading to increased intracellular Aβ accumulation and subsequent neurodegeneration, cell lysis, pouring and deposition of intracellular contents in the brain parenchyma, eventually contributing to amyloid plaques formation [210,211]. Of note, one of the major features in AD is the reduction of α7nAChR protein levels in disease-relevant brain regions such as the cerebral cortex and the hippocampus [212]; this reduction may be explained by the vulnerability of α7nAChR expressing cholinergic neurons to degeneration, as a result of their proneness to Aβ accumulation.

As for glial cells, a significant upregulation of the α7nAChR is observed in AD, probably as a result of a compensatory mechanism established to help protect the neurons from Aβ toxicity [213,214]. Activation of the α7nAChR expressed on glial cells promotes Aβ phagocytosis [215] and - comparably to the periphery - inhibits the release of inflammatory mediators in the CNS. However, as the disease progresses, apoptosis of glial cells overburdened with Aβ contributes to plaque deposition [216] and prolonged exposure to the peptide results in α7nAChR inactivation with subsequent disruption of the anti-inflammatory pathway in the brain [217].

2.3.3 Pharmacological modulation of the cholinergic anti-inflammatory pathway through the α7nAChR

Pharmacological intervention on the cholinergic anti-inflammatory pathway is possible by means of α7nAChR selective agonists. For one, nicotine is able to exert anti-inflammatory effects on peripheral monocytes/macrophages as well as microglia in the CNS. In the periphery, nicotine has proven effective in reducing monocytes/macrophages' pro-inflammatory cytokine production and in restraining their chemotaxis [218,219]. Likewise, nicotine can act as anti-inflammatory agent in the CNS, downregulating microglial activation and the expression of pro-inflammatory cytokines [199]. In light of these premises,

nicotine may be considered to have a protective role against the development of neurodegenerative diseases characterized by a substantial neuroinflammatory response: in AD, for example, nicotine was found to exert a protective effect against Aβ by inhibiting the production of reactive oxygen species from microglial cells [220].

In addition to classic agonists, the α7nAChR can be activated by a class of acetylcholinesterase inhibitors (AChEI), able to act indirectly by either increasing the expression level of the receptor [221] or prolonging the effect of ACh - thereby potentiating its evoked response, and directly as noncompetitive agonists by activating the receptor through interaction with a different binding site from that of ACh [222]. Of note, in light of the role of the α7nAChR in the CAIP, AChEI have also proven effective in modulating the immune response. In the specific context of AD - for which they have been approved as symptomatic treatment - AChEI have also proven able to modulate cytokine production and favor a general shift toward an anti-inflammatory environment. Donepezil, in particular, by inhibiting the activity of AChE, is able to potentiate ACh immunomodulatory effect on lymphomonocytes (PBMC), thereby reducing pro-inflammatory cytokines production [223]. In addition, Donepezil is able to interact directly with the α7nAChR expressed on PBMC and pamper a Th2 mediated immune response, able to induce immunoglobulins production by B lymphocytes. In particular, Donepezil-treated AD patients show significantly higher plasma levels of anti-Aβ42 antibodies compared to those not treated with AChEI or normal controls [109,224].

Chapter 3
DBI/TSPO System

3.1 The translocator protein 18 kDa (TSPO)

The translocator protein 18 kDa (TSPO) is a transmembrane protein widely expressed throughout the body, originally discovered as a peripheral binding site for benzodiazepines. Indeed, after being first discovered in 1977, it was referred to as Peripheral Benzodiazepine Receptor (PBR), to highlight its high affinity for diazepam and to emphasize the difference with the Central Benzodiazepine Receptor (CBR), expressed only in the CNS [225,226].

The protein is encoded by a highly conserved single-copy gene located on chromosome 22q13.3 [227] and consists of 169 aminoacids forming a five α-helices structure, with an intracellular N-terminal and an extracellular C-terminal [228], this last containing a Colesterol Recognition Aminoacid Consensus (CRAC) motif able to recognize and bind cholesterol with high affinity [229].

Although its mRNA is ubiquitously distributed within different tissues, TSPO expression is not homogeneous, probably as a result of a different translational regulation. The highest TSPO levels have been detected in endocrine tissues (such as adrenal glands and gonads), followed by kidney and heart, whereas particularly modest levels are typical of liver and healthy brain [230], where its expression is mainly limited to glial cells [231]. Moreover, TSPO expression has been observed in monocytes [174], platelets and various peripheral blood cells [232-234]. As for the subcellular localization, although TSPO is primarily located on the outer mitochondrial membrane, it has also been identified on the plasma membrane of various cell types [235].

3.2 TSPO physiological functions

TSPO has been implicated in many cellular processes, including control of mitochondrial respiration and function [236], proliferation [237], immunomodulation [238,239] and apoptosis [240]. However, this protein was recognized to be first and foremost a cholesterol transporter - able to transfer cholesterol from the cytoplasm into the mitochondria - and therefore fundamental for cholesterol-dependent steroid synthesis [226,241].

3.2.1 Neurosteroidogenesis

In the CNS, TSPO is considered to have an important role in controlling the rate-limiting step of neurosteroidogenesis, the process of neurosteroids biosynthesis [242]. Those neuroactive molecules are able to exert a physiological neuroprotective effect by binding to membrane-bound receptors for neurotransmitters [243,244] and to regulate a broad spectrum of behavioral functions by acting as either positive or negative modulators of the GABAergic transmission [245]. In particular, neurosteroids interaction with the GABA-benzodiazepine receptor (CBR) - on a different binding site from that of benzodiazepines [246] - has proven able to exert a beneficial effect on behavioral disturbances, while alterations of their levels have been associated to different neuropsychiatric conditions [247]. As a consequence of the physiological role of TSPO, it is possible to infer that its specific ligands have the potential to be used as pharmacological modulators of neurosteroids biosynthesis for the treatment of mood disorders [248].

3.3 TSPO endogenous ligands

In addition to cholesterol, TSPO possesses several endogenous ligands. The best known among them is DBI (diazepam binding inhibitor), a neuropeptide of 11kDa able to inhibit diazepam binding to both central and peripheral benzodiazepine receptors, acting as negative allosteric modulator [249]. DBI expression retraces TSPO distribution, with higher levels in the CNS - particularly in glial cells - and steroidogenic tissues [250]. The proteolytic cleavage of DBI originates small neuroactive peptides, termed endozepines, which retain the ability to modulate GABAergic transmission and stimulate steroids biosynthesis [251,252].

In the CNS, DBI and its metabolites can modulate the GABAergic transmission either through direct binding with the CBR or indirectly by promoting neurosteroidogenesis via TSPO activation [252], playing a consistent role in the development of behavioral disorders [253,254]. In addition, they have been shown to stimulate the synthesis and release of pro-inflammatory cytokines [255] and to induce chemotaxis and phagocytosis in peripheral immune cells [256,257].

3.4 TSPO exogenous ligands

Originally discovered as a peripheral target for benzodiazepines, TSPO sees its function modulated by some of those classical ligands, that are able to promote peripheral monocyte chemotaxis [175] and the

synthesis of neurosteroids [258] through their interaction with the receptor. However, in light of severe side-effects related to benzodiazepine mechanism of action (i.e. sedation, tolerance and abuse), novel selective compounds for the TSPO receptor have been developed to treat these disorders with an improved side-effect profile [259].

Moreover, high selective compounds have also been developed since the '80s to assess TSPO expression through in vitro and in vivo imaging techniques like positron emission tomography (PET) or single-photon emission computed tomography (SPECT) [260,261].

- ***PK11195***

The first non-benzodiazepine synthetic molecule developed to detect TSPO is an isoquinoline carboxamide known as PK11195. This compound is able to penetrate intact blood-brain barrier and to selectively recognize its target - showing no affinity for the CBR - on which it acts as an antagonist [260,262]. In particular, PK11195 was shown to exert anti-inflammatory properties [263], to reduce microglial activation [264] and to interfere with monocytes chemotaxis [175] and with the steroidogenic process [265].

Radiolabelled PK11195 ([^{11}C]PK11195) is now the most widely used specific tracer for TSPO imaging in vivo using PET, although intrinsic limitations of this molecule had recently led to the development of so-called "second-generation" TSPO radioligands [261].

- ***Ro5-4864***

Classical synthetic ligands of TSPO also include the benzodiazepine Ro5-4864 (also known as 4'-clorodiazepam). Similarly to PK11195, this molecule is able to selectively recognize the TSPO - showing no affinity for the CBR; antithetically, however, this compound exerts an agonistic effect on the receptor [262]. Accordingly, Ro5-4864 have been shown to stimulate the synthesis of neurosteroids by increasing cholesterol translocation into the mitochondria [266], to exhibit antidepressant and anxiogenic properties [267,268] and to induce peripheral monocyte chemotaxis [175]. Moreover, the molecule shows a neuroprotective effect [269] and is able to reduce microglial activation and the production of pro-inflammatory cytokines [270].

- ***Emapunil***

Emapunil (also known as XBD-173 or AC-5216) is a selective phenylpurine high-affinity TSPO agonist, able to exert a robust anxiolytic effect in animal models and humans without a direct modulatory effect on the CBR. As a matter of fact, the enhancement of GABAergic neurotransmission mediated by this molecule seems to be induced indirectly through neurosteroidogenesis [271]. More recently, Emapunil was also shown to modulate microglial activation towards an M2 neuroprotective phenotype [272]. Moreover, radiolabeled Emapunil (^{11}C-AC-5216) allows PET imaging and quantification of TSPO in the human brain [273].

3.5 DBI/TSPO system, neuroinflammation and Alzheimer's disease

In the CNS under physiological conditions, TSPO levels are very low; however, they experience a dramatic increase in response to neuroinflammation as a result of glial activation and peripheral monocytes recruitment [274]. This peculiarity has made TSPO a leading biomarker of neuroinflammation and reactive gliosis in a broad spectrum of neuropathological conditions [97].

In Alzheimer's disease patients specifically, DBI has been found upregulated in the CSF and serum [275,276], and TSPO expression has been found significantly increased compared with age-matched healthy controls [277]. In particular, TSPO levels seem to increase in glial cells before the appearance of AD associated brain lesions, and better yet predict subsequent neurodegeneration in the same areas [278]. Furthermore, longitudinal studies suggest that TSPO levels increase over time throughout AD progression from MCI to full-blown dementia [279], and that astrocytes upregulate TSPO earlier in AD pathology compared to microglia [106,280].

TSPO ligands have therefore emerged as potential therapeutic tools for the treatment of AD [281]. In particular, PK11195 and Emapunil have been shown to reduce Aβ accumulation and neuroinflammation [282,283], while Ro5-4864 has proven able to interfere with the development of AD by attenuating Aβ accumulation, reducing gliosis, modulating neurosteroidogenesis [284] and preventing Aβ-induced neurotoxicity [285].

3.6 DBI/TSPO system, neuroinflammation and BPSD

Among different pathological processes involved in AD, neuroinflammation - and possibly the DBI/TSPO system - is especially interesting when looking for biological determinants of BPSD [286], and in particular of the agitation/aggression cluster. The rationale could be the clinical resemblance between agitation/aggression behavioral symptoms and another condition that, similarly to AD, is

characterized by neuroinflammation as its key pathological processes: hyperkinetic delirium [276,287]. Delirium represents an extreme form of sickness behavior, reflecting an exaggerated response of the CNS to systemic inflammatory stimuli [288], and is characterized by elevated DBI serum levels, especially in the hyperkinetic subtype [276]. As aforementioned, DBI levels are also increased in serum and CSF of AD patients [275,276], and it is possible to hypothesize that this can contribute to the genesis of behavioural disturbances via two main mechanisms of action:

- modulation of the GABAergic transmission either through direct binding with the CBR or indirectly by promoting neurosteroidogenesis via TSPO activation [252];
- modulation of peripheral immune cells chemotaxis. It is now well recognized that benzodiazepines often act as precipitants of delirium symptoms in predisposed patients [289]. Given that both benzodiazepines and DBI are able to bind the TSPO receptor expressed on the plasma membrane of peripheral immune cells and promote their chemotaxis [175,256] - thereby contributing to neuroinflammation - it is possible to speculate on the putative involvement of leucocytes chemotaxis in the genesis of behavioral disturbances.

3.7 TSPO polymorphism

The TSPO gene is characterized by a single-nucleotide polymorphism (known as rs6971) in correspondence of exon 2, causing a nonconservative alanine-to-threonine substitution in position 147 [290]. The rs6971 polymorphism results in a conformational change affecting its interaction with a variety of ligands for which affinity had already been demonstrated [291]: the presence of the predominant form (alanine) is associated with high-affinity binding, while the polymorphism (threonine) shows reduced binding affinity [292]. In particular, being located in the close proximity of the CRAC motif, this polymorphism is able to affect cholesterol binding to TSPO receptor [293], thereby reducing steroid production in both heterozygous and homozygous individuals (suggestive of a dominant effect) [294]. Given the role of neurosteroids in mood disorders, a higher frequency of the rs6971 polymorphism has been found in patients suffering from different psychiatric conditions [295,296]. In addition, the polymorphism is able to affect TSPO binding affinity for anxiolytic drugs, thereby reducing their therapeutic effect [297].

Chapter 4
Aim

Alzheimer's disease is an age-related neurodegenerative disorder recognized as the most common cause of dementia [1]. Its etiopathogenesis is far from being completely elucidated and, to date, other pathological processes - beyond the traditional amyloid plaques deposition [30], tau hyperphosphorylation [51] and cholinergic dysfunction [57] - have been recognized to contribute to AD pathogenesis just as much as its pathological hallmarks [63-69]. In particular, neuroinflammation - described in AD since the first characterization of the disorder [84] - is now well recognized as a central feature in its pathogenesis [130]. The resident innate immune cells of the brain are the principal players in neuroinflammation, and their activation leads to a defensive response aimed at promoting Aβ clearance [136]. However, it is now widely accepted that the peripheral immune system is also able to contribute to the neuroinflammatory process taking place in AD by means of phagocytic cells and inflammatory mediators [298], able to access the brain parenchyma through a functionally impaired blood-brain barrier [150] attracted by chemotactic molecules released by activated glial cells [156]. In particular, peripheral monocytes have been demonstrated to cross the BBB in the areas of greater permeability and infiltrate the brain parenchyma, associating with regions of increased Aβ deposition [176]. Nevertheless, their contribution to AD pathogenesis is still controversial: one hypothesis is that, upon recognition of Aβ peptides, infiltrating monocytes differentiate into macrophages and contribute to the clearance of Aβ [173]; however, such conclusion is challenged by studies showing that infiltrating monocytes exhibit reduced differentiation into macrophages and limited phagocytic activity [178].

In light of these premises, principal aim of this PhD project was to investigate the mechanisms underlying peripheral monocytes recruitment in the AD brain and to understand their contribution to Aβ clearance. Secondary goal was to evaluate the disease-modifying potential of three well characterized class of compounds in relation to their ability to influence these processes.

Among the different characteristic pathological features of AD, neuroinflammation is especially interesting also when looking for biological determinants of Behavioral and Psychological Symptoms of Dementia, a wide variety of disturbances usually manifesting during the course the disease [75,286]. In light of the clinical resemblance with hyperkinetic delirium, another condition that is characterized by neuroinflammation as its key pathological process [276,287], the speculation is particularly fitting when referring to the agitation/aggression cluster of BPSD. In particular, is the DBI/TSPO system - in light of its neurosteroidogenic role [252] and involvement in leucocytes chemotaxis [175,256] - to be considered the principal player in the neuroinflammatory process underlying the genesis of behavioural disturbances.

Considering the assumptions above, the concluding aim of this PhD project was to explore the potential implication of neuroinflammation and the DBI/TSPO system in A/A behavioural abnormalities.

4.1 Peripheral monocytes chemotactic response to amyloid β: underlying mechanisms and pharmacological modulation

The first task was to confirm the ability of soluble Aβ to promote peripheral monocytes chemotaxis. To do so, we employed oligomeric Aβ42 as chemotactic stimulus and made a confrontation with the chemotactic power exerted by MCP-1, one of the most effective chemotactic factors for monocytes [166,172].

The mechanisms underlying monocyte chemotactic response to Aβ stimulation were then investigated: in light of its leading role in the recruitment of peripheral immune cells, possible alterations in the expression of CCR2 - the monocytic surface receptor for MCP-1 [299] - has been evaluated.

Parallel investigations focused on the putative role of TSPO, a transmembrane protein identified on the plasma membrane of peripheral monocytes [174] and recognized to be involved in monocytic chemotactic responses [256,257]. The contribution of TSPO to the regulation of Aβ-induced chemotaxis was assessed by evaluating the influence of various selective ligands characterized by different modulatory properties [175,300]. In addition, the modulation of TSPO expression upon stimulation with said ligands was verified. Moreover, in order to speculate on a possible correlation between TSPO chemotactic activity and its rs6971 polymorphism - affecting the interaction with a variety of ligands for which affinity had already been demonstrated [292] - we included the evaluation of Aβ-induced chemotaxis and TSPO expression in monocytes from AD patients characterized by different haplotypes.

Concluding step in this first part of the study was the evaluation of the putative impact on Aβ-induced monocyte migration of some pharmacological and non-pharmacological compounds currently employed/under development for the treatment of AD:

- Donepezil, an acetylcholinesterase inhibitor, effective in modulating the immune response in the specific context of AD [109,224] through its interaction with the α7 nicotinic acetylcholine receptor [221,222];
- Co-ultraPEALut, a nutraceutical compound, showing anti-inflammatory and antioxidant properties on experimental models of AD [126,127];
- Anti-Aβ monoclonal antibodies, able to sequester soluble Aβ in the peripheral blood of AD patients to lower its free level [186].

Moreover, possible alterations in the expression of CCR2 upon stimulation with Donepezil, Co-ultraPEALut and anti-Aβ monoclonal antibodies have been evaluated.

4.2 Peripheral macrophages clearance of Aβ oligomers and its pharmacological modulation

First of all, the phagocytic aptitude of peripheral macrophages in the context of AD pathology had to be determined: to this end, the expression of TREM2 - one of the principal transmembrane receptors involved in the regulation of phagocytosis in myeloid cells [301] - and the circulating level of its soluble form (sTREM2) [302] were assessed.

Secondly, the putative effect on Aβ internalization of the compounds previously selected and tested for their ability to influence peripheral monocyte chemotaxis was evaluated: phagocytosis assays were performed upon stimulation of peripheral macrophages with Donepezil, Co-ultraPEALut and anti-Aβ monoclonal antibodies, using oligomeric Aβ42 as phagocytic target. Moreover, the effect of aforesaid medications on TREM2 expression was also evaluated.

4.3 Neuroinflammation in agitation/aggression behavioral symptoms in Alzheimer's disease: role of the DBI/TSPO system

The first step towards the elucidation of the putative role of the DBI/TSPO system in the pathogenesis of A/A behavioural symptoms in AD was the evaluation of DBI levels in serum and CSF of agitated/aggressive AD patients and their non-agitated/aggressive counterparts.

Subsequently, in light of its role as classical receptor for DBI [249], we analyzed TSPO expression in lymphomonocytes from AD patients dichotomized based on the presence/absence of the A/A symptomatology.

Finally, to investigate the putative involvement of chemotaxis and neuroinvasion in the genesis of A/A, the migration of monocytes from agitated/aggressive AD patients towards oligomeric Aβ42 was quantified.

Thus, the overall goal of this PhD project was to shed light on the contribution of peripheral monocytes to neuroinflammation, a central process in the pathogenesis of Alzheimer's disease and Behavioral and Psychological Symptoms of Dementia, severe and disabling disturbances usually manifesting during the course the disease.

Chapter 5
Materials and Methods

5.1 Patients recruitment

Following Ethical Committee approval and informed consent, AD patients were recruited at the Alzheimer Evaluative Unit of the Neurological Clinic at San Gerardo Hospital (Monza, Italy), with the collaboration of the Neurological Clinic at A. Manzoni Hospital (Lecco, Italy). Patients included in the study had a probable AD diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) and National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria [303] as well as an MMSE $\leq$ 26. Alternative diagnoses were excluded by MR brain imaging and a routine extensive neuropsychological test battery.

Age- and sex-matched healthy controls were recruited among patients' spouses and relatives at the Neurological Clinic of the San Gerardo Hospital (Monza, Italy), following informed consent. The healthy controls had an MMSE > 26 and were available to undergo blood withdrawal. Lack of cognitive impairment and personal or family history of neurological or psychiatric disorders was established by a clinical interview. Cognitive deterioration and previous or on-going neurologic/psychiatric pathologies were considered exclusion criteria.

The exclusion criteria for either patients or controls were:

- positive anamnesis for major cranial trauma
- chronic intestinal inflammatory disease
- hypo- and hyperthyroidism
- kidney or liver insufficiency
- abuse of alcohol, drugs or other substances
- major depressive disorder
- malignant neoplasms
- pharmacological therapy with anti-inflammatory, corticosteroid or immunosuppressive drugs.

The different cohorts of patients recruited for the study are described in Results section.

5.2 Cell lines

Monocytic cell lines THP-1 (derived from acute monocytic leukemia) and U-937 (derived from histiocytic lymphoma) were grown in RPMI 1640 medium supplemented with 10% FBS (Fetal Bovine Serum), 2mM L-glutamine and 1% Penicillin/Streptomycin, at 37°C and 5% CO_2 in a humidified atmosphere.

5.3 Peripheral blood mononuclear cells (PBMC) isolation

For peripheral blood mononuclear cells (PBMC) isolation, whole blood - collected in lithium heparin tubes - was diluted 1:1 with RPMI 1640, stratified on an equal volume of density gradient medium (Lympholyte) and centrifuged at 490xg for 30 min at room temperature (RT). The PBMC were collected at the plasma-Lympholyte interface (buffy coat) and centrifuged in RPMI 1640 at 700xg for 10 min. The resulting pellet was resuspended in physiologic solution (NaCl) and stored at -80 °C for subsequent molecular analysis, or in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin for monocyte isolation.

5.3.1 Monocyte isolation

PBMC resuspended in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin were plated in 6-multiwells plates and incubated for 2 hours at 37°C and 5% CO_2 in a humidified atmosphere to promote monocyte adhesion. After 2 hours, non-adhering PBMC (lymphocytes) were discarded, while adhering monocytes were cultured in RPMI 1640 medium supplemented with 10% FBS (Fetal Bovine Serum), 2mM L-glutamine and 1% Penicillin/Streptomycin, at 37°C and 5% CO_2 in a humidified atmosphere for subsequent functional assays.

5.4 CSF samples preparation

Patients underwent lumbar puncture with a 21-gauge needle to extract CSF (5 ml) which was collected in a polypropylene tube, centrifuged at 2000xg for 10 min at RT and stored at -80 °C.

5.5 Plasma and serum samples preparation

Patients underwent blood withdrawal for serum and plasma separation. After 1 h incubation at 37°C, whole blood without anti-coagulants was centrifuged at 2800xg for 15 min at RT and serum was collected and stored at -80 °C. Whole blood in K3EDTA was centrifuged at 3700xg for 20 min at RT, and plasma was collected and stored at -80 °C.

5.6 Oligomeric Aβ1-42 preparation

Oligomeric Aβ1-42 was prepared according to Stine et al. [304]. Briefly, 200ug of lyophilized Aβ1-42 (Phoenix) were resuspended in 200ul of hexafluoroisopropanol (HFIP) to a final concentration of 1 μg/μl and peptide films were left to dry. After adding 1μl dimethylsulfoxide (DMSO) and 43μl of cold PBS 1X to every 10μg of Aβ - to a final concentration of 50μM - sonication was carried out for 10 minutes in ice. The peptide solution was incubated at 4°C for 24 hours and subsequently centrifuged at 14000 xg at 4°C to recover the oligomers-containing supernatant.

5.7 Immunocomplexes preparation

Immunocomplexes of oligomeric Aβ1-42 and anti-Aβ42 monoclonal antibodies were prepared according to Xiang et al. [305] by incubating oligomeric Aβ1-42 - at a concentration of 125 pM for chemotaxis assay or 15 nM for phagocytosis assay - with anti-Aβ42 monoclonal antibodies (25 ng/ml) for 1 h at 37°C.

5.8 ApoE genotyping

Total DNA was extracted from peripheral blood collected in dipotassium ethylene-diamine-tetraacetic acid (EDTA) using a commercial DNA extraction kit (QIAamp DNA Blood Mini kit, Qiagen) according to the manufacturer instructions. DNA concentration was determined spectrophotometrically at 260 nm and amplification was performed using specific primers. To analyze apolipoprotein E (ApoE) genotype, the amplification products were digested with HhaI and subjected to electrophoresis on 4% agarose gels. Each ApoE isoform was distinguished by a unique combination of HhaI fragment sizes [306].

5.9 μ-slide chemotaxis assay

THP-1 cells were suspended in gel matrix (Cultrex BME) to reach the final concentration of 3×10^6 cells/ml. 6 μl of the suspension were loaded in the central channel of the μ-slide chamber (Ibidi®, Gräfelfing, Germany) and set aside until the gel was solid. Later, the lateral chambers were filled with 15 μL of RPMI 1640 medium supplemented with 2% FBS alone or in combination with chemoattractant

(either MCP-1 10 ng/ml or Aβ 125 pM). Cellular migration was recorded for 24 hours by means of time-lapse microscopy and the migratory trajectories of 20 cells/chamber were designed using the "Chemotaxis and Migration Tool" (Ibidi®). The Forward Migration Index (FMI, parallel component of the chemotactic gradient) was used to measure cellular motility (higher migration rates correspond to higher FMI values).

5.10 Boyden chambers chemotaxis assay

Boyden chambers (Corning™ Transwell™) were used to perform these experiments. 600 µl of RPMI 1640 medium supplemented with 2% FBS alone or in combination with chemoattractants were loaded in the lower chamber of each well. Adherent monocytes were resuspended in RPMI 1640 medium supplemented with 2% FBS to a final concentration of 5×10^5 cells/ml; 100 µl were seeded in the upper chamber of the Boyden system, on a porous membrane filter - with pore diameter of 5 µm - positioned between the upper and the lower compartments. Equivalently, 100 µl of THP-1 or U-937 cells at a final concentration of $8x10^5$ cells/ml, suspended in RPMI 1640 medium supplemented with 2% FBS, were seeded on a porous membrane with pore diameter of 8 µm. Cells were allowed to migrate for 3 hours at 37 °C and 5% CO_2 in a humidified atmosphere. After the incubation, the filters were fixed, colored with a modified-Giemsa staining, removed from the inserts and mounted on glass slides; the cells attached to the porous membrane were photographed at the optical microscope (magnification 10x) and the number of cells in 20 random fields/filter was counted. At the same time, cells migrated through the porous filter into the lower compartment of the Boyden chamber were counted using a hemocytometer. The results were combined and the chemotactic index was expressed as a ratio between the number of cells migrated toward the stimulus and the number of cells migrated in absence of any stimuli.

5.11 Phagocytosis assay

Adherent monocytes were resuspended in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin to a final concentration of 8×10^5 cells/ml, plated on coverslips in 12-multiwells plates and differentiated in macrophages for 6 days at 37 °C and 5% CO_2 in a humidified atmosphere, in presence of macrophages colony-stimulating factor (M-CSF) 50 ng/ml. Equivalently, U-937 cells were resuspended in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin to a final concentration of 5x105 cell/ml, plated on coverslips in 12-multiwells plates and differentiated in macrophages for 3 days at 37 °C and 5% CO_2 in a humidified

atmosphere, in presence of phorbol-12-myristate 13-acetate (PMA) 200 nM. After differentiation, macrophages have been incubated with fluorescent oligomeric Aβ42 15 nM (Hilyte Fluor 488-labeled, AnaSpec Inc.) - alone or in combination with ancillary stimuli - for 24 hours at 37 °C and 5% CO_2 in a humidified atmosphere. Coverslips were fixed in 10% formalin, incubated for 1 hour at RT with Phalloidin-Atto 550 (Merk, Darmstadt, Germany) and mounted on slides with mounting medium added with DAPI. 5 random fields/coverslip were examined using an inverted microscope equipped for epifluorescence (Axio Observer 200, Zeiss, Oberkochen, Germany) and 64 digital images/field were obtained. Digital images have been processed with Fiji (ImageJ) software and fluorescent Aβ internalization has been expressed as a ratio between integrated density of fluorescence and the number of nuclei.

5.12 Real-time PCR

THP-1 or U-937 cells - differentiated or not into macrophages - were plated at a concentration of 2×10^6 cells/ml in 6-multiwells plates in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin in presence of relevant stimuli, incubated at 37 °C and 5% CO_2 in a humidified atmosphere for an appropriate amount of time and pelleted after incubation. On the other hand, human PBMC pellet was used as it is. Total RNA was extracted from cellular pellet using the RNeasy Mini kit (Qiagen), according to the manufacturer instructions. RNA concentration was determined spectrophotometrically at 260 nm. cDNA was synthesized from RNA using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen by Life Technologies Carlsbad, CA, USA) at the following conditions: 10 min at 25 °C and 60 min at 42 °C. The reaction was terminated at 85°C for 5 min.

Primers used for Real-time PCR were: KiCqStart™ Primers pre designed by Sigma Aldrich (Merck KGaA, Darmstadt, Germany) for CCR2, TREM2 and β-actin, or TaqMan probes (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, US) for TSPO and β-actin. Samples were assayed using the ABI Prism 7500 HTSequence Detection System (Applied Biosystems). For the quantification of TSPO, mRNA TaqMan Gene expression kit (Thermo Scientific) was used under these conditions 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 20 s. For the quantification of the other targets, Titan HotTaq EvaGreen® qPCR Mix (ROX, Bioatlas, Tartu, Estonia) was used at the following conditions: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 15 min, 40 cycles at 95 °C for

15 s, 58 °C for 20 s. For relative quantification of target gene mRNA versus housekeeping gene (β-actin) mRNA, the comparative CT (cycle threshold) method was used.

5.13 Western Blot

THP-1 or U-937 cells - differentiated or not into macrophages - were plated at a concentration of 3×10^6 cells/ml in 6-multiwells plates in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin in presence of relevant stimuli, incubated at 37 °C and 5% CO_2 in a humidified atmosphere for an appropriate amount of time and pelleted after incubation. On the other hand, human PBMC pellet was used as it is. The lysis of cellular pellets was conducted on ice in cell extraction buffer (Invitrogen) supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich). Lysates were sonicated and centrifuged at 21,000xg for 10 min at 4 °C to pellet insoluble material. The pellet was discarded and the total protein concentration in the supernatant was determined by Bradford's method using UV-1601 spectrophotometer (Shimadzu).

To allow electrophoretic separation, 50 μg proteins were resuspended in loading buffer (40 mM Tris pH 6.8, glycerol 6%, β-mercaptoethanol 1.25%, SDS 1%, bromophenol blue) and denatured for 10 minutes at 100°C. Samples were resolved on a 12% polyacrylamide gel for 2 hours at 100V in Running Buffer 1X (glycine 14.5 g/l, Tris 3 g/l, SDS 1 g/l) and then transferred to nitrocellulose. Electrophoretic wet transfer was performed for 90 min at 140 mA in a specific transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol pH 8.5). Membranes were firstly saturated with blocking solution (Tris Buffered Saline - Tween added with 5% powder milk) and then probed over-night at 4°C with primary antibodies against the specific target diluted in blocking solution. For densitometry analysis purposes, the filter was also probed in a second step with primary antibodies against housekeeping proteins. Membranes were washed three times with Tris Buffered Saline - Tween solution (50 mM Tris-Cl, 150 mM NaCl; pH 7.6 + Polyoxyethylene sorbitane monolaureate 0.1%) for 30 minutes and incubated for 1 hour with HRP-conjugated secondary antibodies diluted in blocking solution. Antibody-specific signals were detected using an enhanced chemiluminescent detection system (ECL, EuroClone) using ImageQuant™ LAS 4000 and densitometric assessment of band intensities was performed using ImageJ software.

Antibodies used for immunoblot were as follows: monoclonal rabbit anti-CCR2 (Cell Signaling Technology; 1:1000; O/N at 4°C) followed by HRP-linked anti-rabbit IgG (Sigma; 1:8000; 1 h at RT): monoclonal goat anti-TSPO (1:500; O/N at 4°C) followed by HRP-linked anti-goat IgG (Sigma; 1:8000;

1 h at RT); polyclonal goat anti-TREM2 (Thermo Fisher Scientific, 1:1000; O/N at 4°C) followed by HRP-linked anti-goat IgG (Sigma; 1:8000; 1 h at RT); monoclonal mouse anti-βtubulin (R&D Systems; 1:5000; 1 h at RT) or monoclonal mouse anti-βactin (Sigma-Aldrich; 1: 40.000; 1 h at RT) followed by HRP-linked anti-mouse IgG (Sigma-Aldrich; 1: 20.000; 1 h at RT) as loading controls.

5.14 DBI Enzyme-Linked Immunosorbent Assay

Serum and CSF DBI concentrations were measured using a commercially available kit (human DBI (ACBP) ELISA kit - AbFrontier). The samples were previously diluted 1:60 (serum) and 1:100 (CSF) in sample diluent and the assay was performed according to the manufacturer instructions. Briefly, samples were loaded into the plate wells, incubated (2 h, RT) and washed 3 times before adding the primary antibody (1 h, RT). After 3 washes, they were incubated with the secondary antibody (30 min, RT), washed 3 times and incubated with TMB to start the colorimetric reaction, which was stopped using the stop solution provided by the kit. DBI concentration was determined spectrophotometrically at 450 nm using FLUOstar Omega (BMG LABTECH) and it was quantified according to the standard curve.

5.15 Aβ42 Enzyme-Linked Immunosorbent Assay

Aβ42 concentration has been quantified in the culture medium of U-937 macrophages - treated with Donepezil (100 ng/ml) and/or pre-treated with MLA - undergoing the phagocytosis assay, using a commercially available kit (Innotest β-amyloid (1-42) HS Conj, Fujirebio) according to the manufacturer instructions. Briefly, samples were loaded into the plate wells, incubated (3 h, RT) and washed 5 times before adding the anti-Aβ (1-42) IgG labelled with biotin (1 h, RT). After 5 washes, they were incubated with the HRP-labelled streptavidin (30 min, RT), washed 5 times and incubated with HRP substrate to start the colorimetric reaction, which was stopped using the stop solution provided by the kit. Aβ42 concentration was determined spectrophotometrically at 450-690 nm using FLUOstar Omega (BMG LABTECH) and it was quantified according to the standard curve.

5.16 soluble TREM2 Enzyme-Linked Immunosorbent Assay

Plasma soluble TREM2 concentration has been measured using a commercially available kit (ab224881 Human TREM2, Abcam) according to the manufacturer instructions. Briefly, samples - previously diluted 1:80 - were loaded into the plate wells and incubated with the Antibody Cocktail, containing both capture and detector antibodies (1 h, RT). After 3 washes, they were incubated with TMB to start the

colorimetric reaction, which was stopped using the stop solution provided by the kit. sTREM2 concentration was determined spectrophotometrically at 450-570 nm using FLUOstar Omega (BMG LABTECH) and it was quantified according to the standard curve.

5.17 Statistical analysis

Data are shown as mean ± standard deviation (SD). Statistical analysis was performed with GraphPad Prism 7. Data are expressed throughout the study as mean ± standard deviation (SD). Two-tailed Student's t test was used for computing difference between two groups, while analysis of variance (ANOVA) was used as appropriate for three or more groups, followed by either Tukey or Bonferroni Multiple comparison pot hoc test. Differences were considered significant only for $p<0.05$. Values that differ by two standard deviations from the mean value of each group were excluded from the analysis.

Chapter 6
Results

A. Recruited populations

6.A.1 Chemotaxis and phagocytosis

Chemotaxis and phagocytosis assays were performed on a totality of 34 Alzheimer's disease patients diagnosed according to the DSM-V and NINCDS/ADRDA criteria. Complete clinical, demographic and social information about the recruited populations are listed in **table 1.**

AD patients n=34					
Sex, M%, F%	14 (41%); 20 (59%)				
Age (years)	70 ± 9 (52-87)				
MMSE (score)	21.3 ± 5.1				
ApoE ε4 genotype (%)	**2/2**	**2/3**	**3/3**	**3/4**	**4/4**
	0%	4%	57%	32%	7%

Table 1. AD: Alzheimer's disease; range intervals in brackets; values expressed as mean ± SD.

6.A.2 DBI/TSPO and CCR2 assessment

The role of TSPO and CCR2 in the regulation of Aβ-induced chemotaxis in the context of AD pathology was investigated on a recruited population of 60 Alzheimer's disease patients - diagnosed according to the DSM-V and NINCDS/ADRDA criteria - and 30 non-demented healthy controls, matched with the diseased subjects for age and sex. Complete clinical, demographic and social information about AD and CTRL recruited populations are listed in **table 2**.

	AD n=60					CTRL n=30
Sex, M%, F%	25 (39%); 35 (61%)					14 (47%); 16 (53%)
Age (years)	78 ± 5 (90-67)					79 ± 6 (66-86)
Level of education (years)	6.0 ± 2.7 (3-13)					N/P
Disease duration (months)	39.8 ± 22.5 (4-9)					N/A
MMSE (score)	17.9 ± 5.3					> 26
NPI-12 (score)	18.5 ± 2.3 (0-86)					N/A
AA+ (n, %)	25 (40%)					N/A
AChEI therapy (n, %)	27 (45%)					N/A
Memantine therapy (n, %)	21 (35%)					N/A
ApoE genotype (%)	**2/2**	**2/3**	**3/3**	**3/4**	**4/4**	N/P
	2%	5%	39%	44%	10%	

Table 2. AD: Alzheimer's disease; **CTRL:** controls: **N/A:** not applicable; **N/D:** not determined; range intervals in brackets; values expressed as mean ± SD.

To elucidate the putative role of the DBI/TSPO system in the pathogenesis of agitation/aggression behavioural symptoms in AD, we made use of a subgroup of subjects - identified in the present AD population - displaying this disturbance: based on the specific agitation/aggression (AA) score (frequency × severity) extrapolated from the NPI-12, AD patients were dichotomized in AA+ (AA score > 0) and AA- (AA score = 0). Complete clinical, demographic and social information about AA+ and AA- patients in the recruited AD population are listed in **table 3**.

	AD AA+ n=24	AD AA- n=36
Sex, M%	13 (53%)	20 (55%)
Age (years)	78.7 ± 6 (69-85)	77.9 ± 4 (67-70)
Level of education (years)	5.5 ± 2.0 (3-13)	6.3 ± 2.4 (3-13)

Disease duration (months)	46.5 ± 27.0 (12-92)	34.5 ± 21.0 (10-96)
MMSE (score)	16.0 ± 6.4 (4-24)	18.6 ± 4.8 (9-26)
NPI-12 (score)	37.17 ± 16.67 (16-86)	13.48 ± 12.42 (0-46)
AA (score)	6.1 ± 3.2 (1-12)	N/A
AChEI therapy (%)	75%	72%
Memantine therapy (%)	32%	38%

Table 3. AA+: agitated/aggressive AD patients; **AA-:** not agitated/aggressive AD patients; **N/A:** not applicable; range intervals in brackets; values expressed as mean ± SD.

Moreover, monocytes from a subset of AD patients and controls belonging to the present population was employed to perform chemotaxis assays.

6.A.3 TREM2 assessment

The role of TREM2 in the modulation of Aβ phagocytosis was evaluated on a recruited population of 48 Alzheimer's disease patients - diagnosed according to the DSM-V and NINCDS/ADRDA criteria – and 20 non-demented healthy controls, matched with the diseased subjects for age and sex. For the purpose of the study AD patients were divided according to Donepezil treatment. Complete clinical, demographic and social information about AD and CTRL recruited populations are listed in **table 4.**

	AD no treatment n=20	**AD DNPZ treatment n=28**	**CTRL n=20**
Sex, M%, F%	11 (55%); 9 (45%)	8 (29%); 20 (71%)	7 (35%); 13 (65%)
Age (years)	77 ± 6 (67-90)	78 ± 7 (59-89)	73 ±5 (61-78)
MMSE (score)	17.5 ± 7.3	18.9 ± 3.8	> 26
AChEI therapy duration (months)	N/A	11.5 ± 10.5 (2-36)	N/A
ApoE ε4 genotype (%)	65%	48%	14%

Table 4. AD: Alzheimer's disease; **CTRL:** controls: **DNPZ:** Donepezil; **N/A:** not applicable; range intervals in brackets; values expressed as mean ± SD.

B. Monocyte chemotaxis

6.B.1 Aβ-induced monocyte chemotaxis

To evaluate the ability of Aβ - the principal protein accumulated in the AD brain - to promote peripheral monocytes chemotaxis, we made use of monocytic cell lines and human monocytes, and employed two specific chemotaxis assays. For this purpose, oligomeric Aβ42 was used at a concentration in the same order of magnitude as the pathological cut-off value in human CSF (125 pM) [307].

- **Cellular migration in μ-slide chemotaxis chambers**

Preliminary studies were performed on THP-1 cell lines using μ-slide chambers as chemotactic apparatus. To promote migration, THP-1 cells were exposed to either oligomeric Aβ42 125 pM or MCP-1 10 ng/ml, the most powerful chemotactic stimulus for monocytes, chosen as a positive control. Time-lapse microscopy showed that Aβ was able to attract THP-1 cells to the same extent as MCP-1, producing a two-fold increase in FMI compared to basal conditions (basal 0.18 ± 0.04 vs. Aβ 0.36 ± 0.02 vs. MCP-1 0.35 ± 0.04; $p<0.01$ Aβ vs. basal; $p<0.01$ MCP-1 vs. basal) (**Figure 1**).

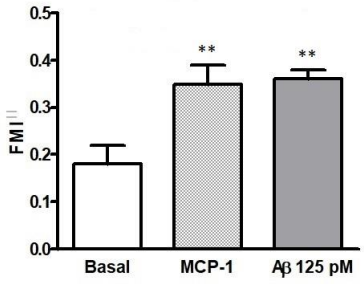


Figure 1: THP-1 cell migration increases after stimulation with MCP-1 (n=5; $p<0.01$ MCP-1 vs. basal; $p<0.01$ at ANOVA, followed by Tukey's multiple comparisons test; mean ± SD) or Aβ ($p<0.01$ Aβ vs. basal).

- **Cellular migration in Boyden chambers**

In parallel with monocytic cell lines migration experiments, the effect of oligomeric Aβ42 (125 pM) and MCP-1 (10 ng/ml) was tested on human monocytes from healthy controls using Boyden Chambers

chemotaxis assay. Human monocytes demonstrated a significant increase in chemotaxis towards Aβ respect to basal conditions (Aβ 4.41 ± 1.88; $p<0.05$ Aβ vs. basal), even though the effect resulted less evident respect to MCP-1 stimulated monocytes (MCP-1 19.47 ± 3.33; $p<0.01$ MCP-1 vs. basal) (**Figure 2**).

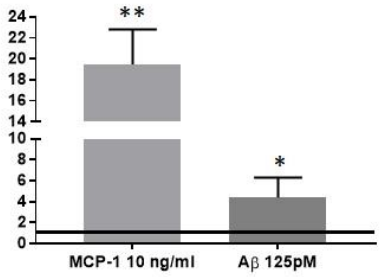


Figure 2: Human monocytes chemotaxis towards Aβ increases by 150% compared to basal conditions (n=6; $p<0.05$ Aβ vs. basal; $p<0.01$ at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD). The effect results less evident respect to MCP-1, boosting monocytes migration by 900% ($p<0.01$ MCP-1 vs basal). Basal (horizontal line) equals a chemotactic index of 1.

To highlight any potential difference, the chemotactic behaviour of monocytes collected from AD patients was evaluated by comparison with that of monocytes from age-matched healthy controls. Results showed that Aβ is able to induce a stronger chemotactic response in monocytes from AD patients compared to controls (AD 4.4 ± 0.6 vs. CTRL 2.0 ± 0.4, $p<0.01$ AD vs. CTRL) (**Figure 3**).

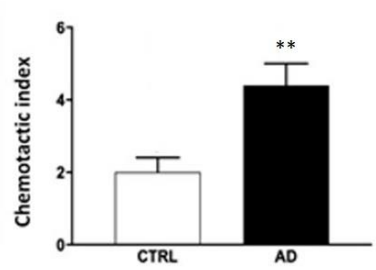


Figure 3: Monocytes from AD patients (n=8) show an increase in Aβ-induced chemotaxis respect to controls (n=6; $p<0.01$ at unpaired two-tailed t-test, mean ± SD).

6.B.2 Regulation of Aβ-induced monocyte chemotaxis: CCR2 expression

The mechanisms underlying monocyte chemotactic response to Aβ stimulation was investigated. In light of its leading role in the recruitment of peripheral immune cells, possible alterations in the expression of CCR2 has been evaluated by Real-time PCR and Western blot analysis.

- **Aβ-induced CCR2 expression**

CCR2 mRNA and protein expression levels were quantified after oligomeric Aβ42 125 pM treatment in THP-1 monocytic cell lines; mRNA expression was also quantified in lymphomonocytes (PBMC) from control subjects and AD patients. The results showed that Aβ was not able to modify mRNA and protein expression levels in THP-1 cells; moreover, no difference was observed in mRNA levels of CCR2 in PBMC from AD patients and controls (AD 0.9 ± 0.7 vs. CTRL 1.2 ± 0.8) (**Figure 4**).

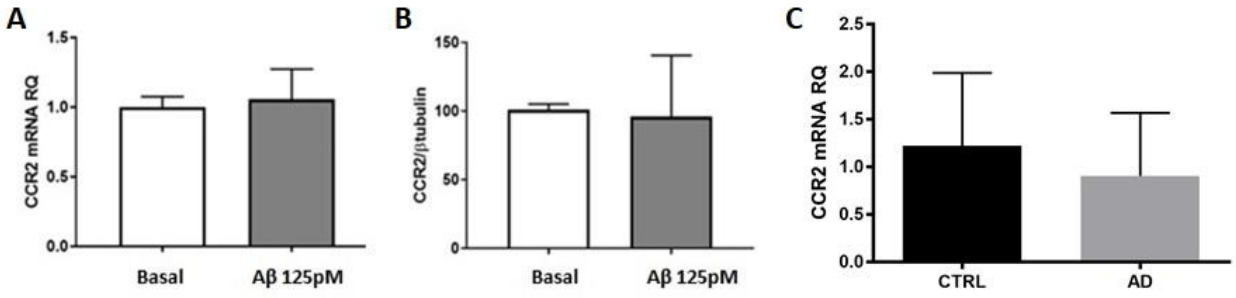


Figure 4: CCR2 mRNA **(A)** and protein **(B)** expression levels in THP-1 cells do not change upon exposure to Aβ (n=5; p>0.05 at unpaired two-tailed t-test; mean ± SD). **(C)** PBMC from AD patients (n=18) and controls (n=10) show no difference in terms of CCR2 mRNA levels (p>0.05 at unpaired two-tailed t-test; mean ± SD).

6.B.3 Regulation of Aβ-induced monocyte chemotaxis: the role of TSPO

Parallel investigations focused on the putative role of TSPO, another transmembrane receptor recognized to be involved in monocytic chemotactic responses.

6.B.3.1 Monocyte chemotaxis

The contribution of TSPO to the regulation of Aβ-induced chemotaxis was assessed through Boyden Chamber experiments by evaluating the influence of different selective ligands.

- **TSPO agonists: Emapunil and Ro5-4864**

The ability of TSPO agonists - Emapunil and Ro5-4864 - to influence monocyte chemotaxis was tested in both THP-1 monocytic cell lines (Emapunil) and human monocytes (Ro5-4864). Experiments performed on cell lines with two different concentrations of the compound (1 µM and 50 µM) showed that Emapunil 1µM is able to induce an increase of 65% of THP-1 chemotactic properties (Emapunil 1 µM 1.7 ± 0.6; $p<0.05$ Emapunil 1 µM vs. basal) (**Figure 5**).

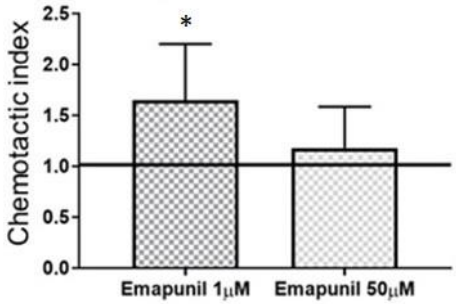


Figure 5: Emapunil 1 µM significantly increases THP-1 cells chemotactic properties (n=6; $p<0.05$ Emapunil 1µM vs basal; $p<0.05$ at ANOVA followed by Tukey's multiple comparisons test; mean ± SD). Basal (horizontal line) equals a chemotactic index of 1.

Furthermore, a significant increase of migration was observed in human monocytes by means of Ro5-4864. In particular, Ro5-4864 10 µM was able to induce chemotaxis in both monocytes from healthy controls (Ro5-4864 10 µM 2.1 ± 0.7; $p<0.05$ Ro5-4864 10 µM vs. basal) and AD patients (Ro5-4864 10 µM 1.8 ± 0.4; $p<0.05$ Ro5-4864 10 µM vs. basal) (**Figure 6**).

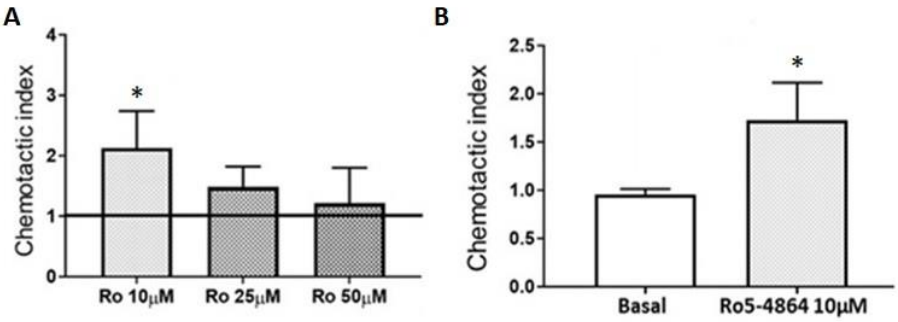


Figure 6: (A) Ro5-4864 10 µM stimulation increases by 110% the chemotactic index of human monocytes from healthy controls (n=5; $p<0.05$ Ro5-4864 10 µM vs. basal; $p<0.05$ at ANOVA followed by Tukey's multiple comparison test; mean ± SD) **(B)** and by 70% in AD patients (n=5; $p<0.05$ Ro5-4864 10 µM vs. basal at unpaired two-tailed t-test; mean ± SD). Basal (horizontal line) equals a chemotactic index of 1.

- **TSPO antagonist: PK11195**

Inhibitory properties of PK11195 were also evaluated: THP-1 monocytic cell lines and monocytes from AD patients were stimulated with PK11195 at different concentrations (20 μM and 120 μM), alone or in combination with oligomeric Aβ42 (125 pM). Results show that PK11195 alone is not able to alter monocytic chemotactic behaviour respect to basal conditions, but partially reverted Aβ-induced chemotaxis in a concentration dependent manner, in both THP-1 cells (Aβ 3.8 ± 2.9 vs. Aβ+PK 20μM 1.7 ± 0.8 vs. Aβ+PK 120μM 1.5 ± 0.7; $p<0.01$ Aβ+PK 20μM vs. Aβ; $p<0.001$ Aβ+PK 120μM vs. Aβ) and monocytes from AD patients (Aβ 4.9 ± 0.8 vs. Aβ+PK 20μM 2.9 ± 1.2 and vs. Aβ+PK 120μM 1.0 ± 0.3; $p<0.05$ Aβ+PK 20μM vs. Aβ; $p<0.001$ Aβ+PK 120μM vs. Aβ) (**Figure 7**).

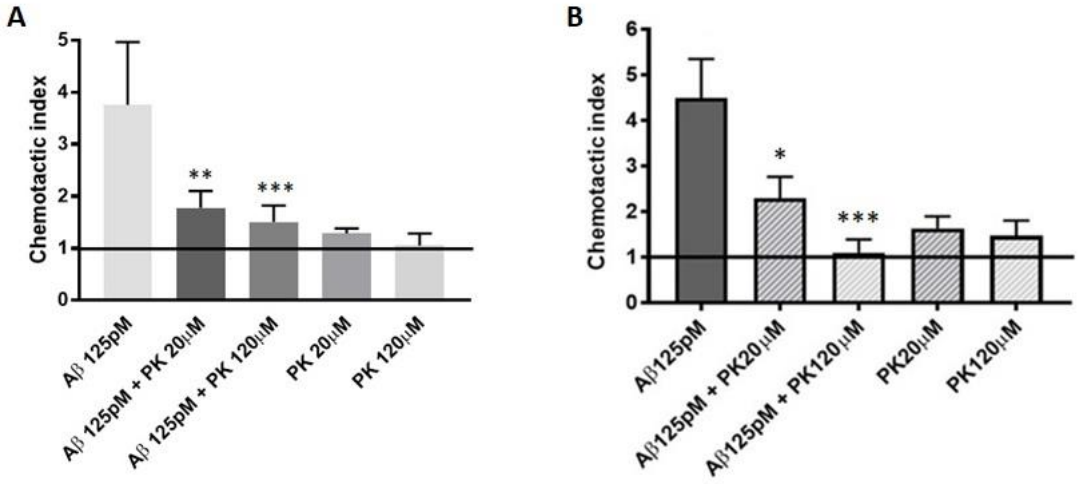


Figure 7: (A) PK11195 alone doesn't alter monocytic chemotactic behavior respect to basal, but partially reverts Aβ-induced migration in a concentration dependent manner in both THP1 monocytic cell lines (n= 6; $p<0.01$ Aβ+PK 20μM vs. Aβ; $p<0.001$ Aβ+PK 120μM vs. Aβ; $p<0.01$ at ANOVA followed by Tukey's multiple comparisons test; mean ± SD) **(B)** and human monocytes from AD patients (n=5; $p<0.05$ Aβ+PK 20μM vs. Aβ; $p<0.001$ Aβ+PK 120μM vs. Aβ; $p<0.05$ at ANOVA followed by Tukey's multiple comparisons test; mean ± SD). Basal (horizontal line) equals a chemotactic index of 1.

6.B.3.2 TSPO expression

To verify if differences in monocyte chemotactic behavior could be dependent on the modulation of TSPO expression, Real-time PCR and Western blot were performed to evaluate mRNA and protein levels upon stimulation with oligomeric Aβ and/or TSPO selective ligands.

- **Aβ-induced TSPO expression**

TSPO mRNA and protein expression levels were quantified after oligomeric Aβ42 125 pM treatment in THP-1 monocytic cell lines. TSPO mRNA expression was also quantified in PBMC from control subjects and AD patients. The results showed that Aβ was not able to modify TSPO mRNA and protein expression levels in THP-1 cells; moreover, no difference was observed in mRNA levels of TSPO in PBMC from AD patients and controls (CTRL 1.70 ± 2.70 vs. AD 1.30 ±1.0) (**Figure 8**).

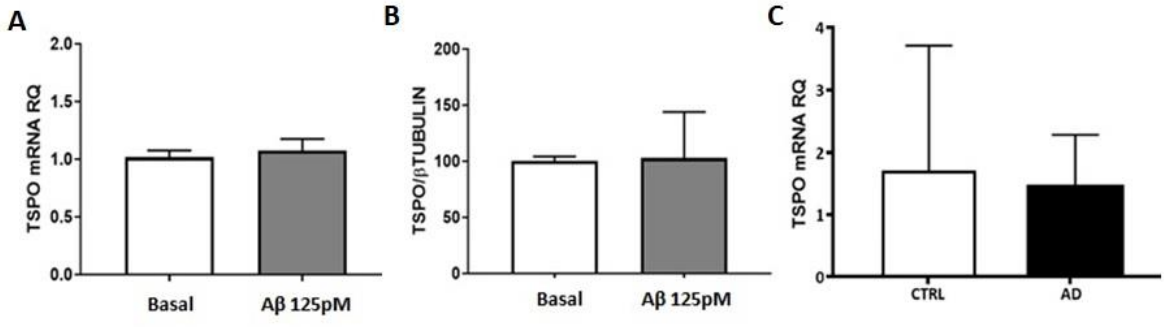


Figure 8: TSPO mRNA **(A)** and protein **(B)** expression levels in THP-1 cells do not change upon exposure to Aβ (n=5; p>0.05 at unpaired two-tailed t-test; mean ± SD). **(C)** PBMC from AD patients (n=60) and controls (n=30) show no difference in terms of TSPO mRNA levels (p>0.05 at unpaired two-tailed t-test; mean ± SD).

- **TSPO expression induced by selective ligands**

TSPO mRNA and protein expression levels were quantified upon stimulation of THP-1 cells with selective agonists (Emapunil 1 μM and Ro5-4864 10 μM) or antagonist (PK11195, at different concentrations) alone or in combination with oligomeric Aβ42 125 pM. As for Emapunil and Ro5-4864, no difference in mRNA levels of TSPO were observed after stimulation with either selective agonists alone or in combination with Aβ, compared to basal condition (**Figure 9**).

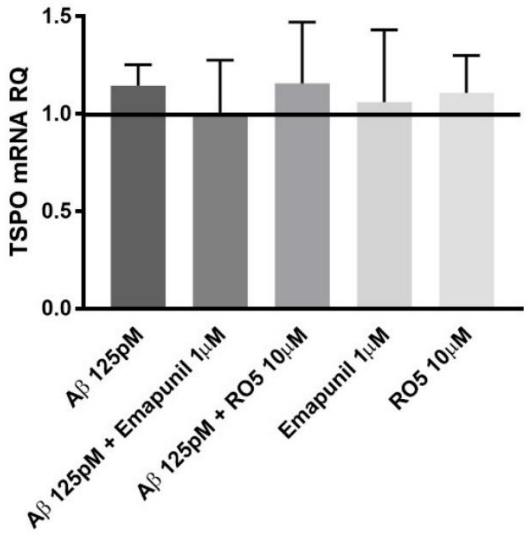


Figure 9: Emapunil and Ro5-4864, alone or in combination with Aβ, do not have an impact on TSPO mRNA expression levels in THP-1 monocytic cell lines (n=4; p>0.05 at ANOVA followed by Tukey's multiple comparisons test; mean ± SD). Basal (horizontal line) equals a TSPO mRNA RQ of 1.

At the same time, none of the concentration used of PK11195 - alone or in combination with Aβ - were able to modify TSPO mRNA or protein expression levels, that remained similar to basal conditions (**Figure 10**).

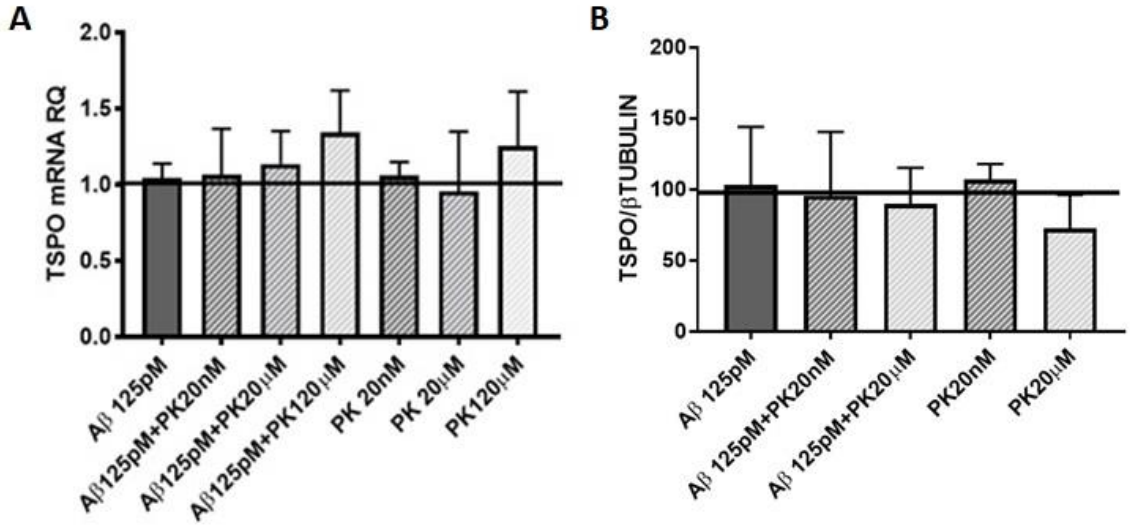


Figure 10: (A) PK11195, alone or in combination with Aβ, does not change TSPO mRNA expression levels in THP-1 cells (n=4; p>0.05 at ANOVA followed by Tukey's multiple comparisons test; mean ± SD); basal (horizontal line) equals a TSPO mRNA RQ of 1. **(B)** Similarly, TSPO protein expression levels do not change upon exposure to PK11195, alone or in

combination with Aβ (n=4; p>0.05 at ANOVA followed by Tukey's multiple comparisons test; mean ± SD); basal (horizontal line) equals a percentage TSPO/βTUBULIN ratio of 100.

6.B.3.3 TSPO polymorphism

A possible correlation between monocyte chemotactic modulation and rs6971 TSPO polymorphism was explored.

- **Monocyte chemotaxis**

Chemotaxis induced by oligomeric Aβ42 125 pM was evaluated by Boyden chamber assay in monocytes from AD patients divided basing on their haplotype (unfortunately, none of the patients considered showed G/A polymorphism). Results didn't show any difference in chemotactic activity between different haplotypes (G/G 3.8 ± 1.1 vs. A/A 3.3 ± 1.3) (**Figure 11**).

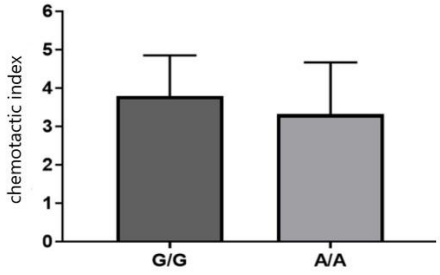


Figure 11: Monocytes from AD patients divided for TSPO polymorphism do not show any difference in chemotactic activity (n=5; p>0.05 at unpaired two tailed t-test; mean ± SD).

- **TSPO expression**

TSPO mRNA levels have been quantified in PBMC from AD patients divided basing on their aplotype. Real-time PCR didn't show any difference in terms of mRNA expression between different aplotypes (G/G 1.4 ± 1.1 vs. G/A 1.4 ± 1.9 vs. A/A 1.8 ± 1.8) (**Figure 12**).

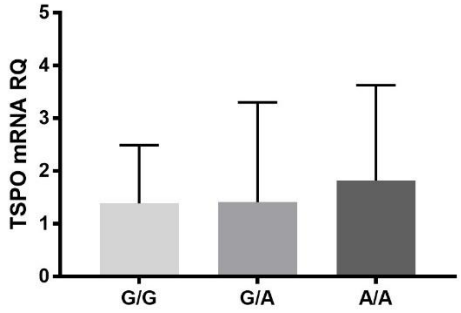


Figure 12: PBMC from AD patients divided for TSPO polymorphism (G/G n=18; G/A n=13; vs. A/A n=4;) do not show any difference in terms of mRNA expression ($p>0.05$ at ANOVA followed by Tukey's multiple comparisons test; mean ± SD).

6.B.4 Modulation of Aβ-induced monocyte chemotaxis

The putative impact on Aβ-induced monocyte migration of some pharmacological and non-pharmacological compounds currently employed/under development for the treatment of AD was assessed through Boyden chamber chemotaxis assay using both monocytic cell lines and human monocytes.

- ***Donepezil***

The putative immunomodulatory effect of Donepezil - in virtue of its interaction with α7nAchR - on peripheral monocytes chemotactic properties has been evaluated on human monocytes from AD patients and healthy controls. Oligomeric Aβ42 125 pM was used as chemoattractant; in addition, monocytes were stimulated with Donepezil (50 and 100 ng/ml for controls; 100 ng/ml for AD) and/or pre-treated with α7nAChR inhibitor methyllycaconitine (MLA 1μM). Of note, Donepezil was used at concentrations mirroring the plasma steady-state concentrations achieved in AD patients treated with a daily dose of 10 mg (50 ng/ml) or 23 mg (100 ng/ml) respectively [308]. Results showed inhibition of Aβ-induced migration in human monocytes from healthy controls using both Donepezil concentrations (Aβ 2.9 ± 1.2 vs. Aβ+DNPZ 50ng/ml 1.1 ± 0.4 vs. Aβ+DNPZ 100ng/ml 0.7 ± 0.3; $p<0.001$ Aβ+DNPZ 50ng/ml vs. Aβ; $p<0.001$ Aβ+DNPZ 100ng/ml vs. Aβ). Moreover, Donepezil 100ng/ml significantly inhibited Aβ-induced migration in human monocytes from AD patients (Aβ 3.0 ± 2.6 vs. Aβ+DNPZ 100ng/ml 1.4 ± 0.6; $p<0.05$ Aβ+DNPZ 100ng/ml vs. Aβ). MLA was not able to interfere with Donepezil-induced chemotactic inhibition in either AD patients or controls (**Figure 13**).

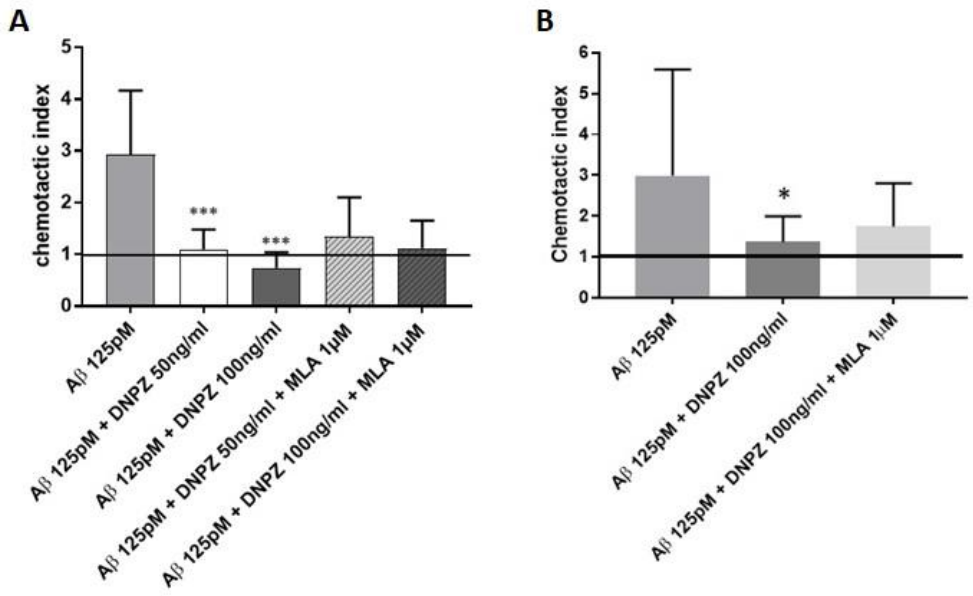


Figure 13: (A) Donepezil significantly inhibits Aβ-induced migration in human monocytes from healthy controls (n=4 $p<0.001$ Aβ+DNPZ 50ng/ml vs. Aβ; $p<0.001$ Aβ+DNPZ 100ng/ml vs. Aβ; $p<0.001$ at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD). **(B)** Aβ-induced migration is inhibited in human monocytes from AD patients (n=7) by means of Donepezil 100ng/ml ($p<0.05$ Aβ+DNPZ 100ng/ml vs. Aβ; $p<0.05$ at ANOVA, followed by Sidak's multiple comparisons test, mean ± SD). Basal (horizontal line) equals a chemotactic index of 1.

- **Donepezil modulation of Aβ-induced CCR2 expression**

The effect of Donepezil on CCR2 mRNA and protein expression levels has been evaluated in U-937 cells, monocytic cell lines endogenously expressing the α7nAChR. Stimulation was carried out using oligomeric Aβ42 125 pM, alone or in combination with Donepezil 100 ng/ml and/or MLA 1μM as pre-treatment. Both Real-time PCR and Western blot analysis showed that Donepezil is not able to alter CCR2 mRNA and protein expression levels (**Figure 14**).

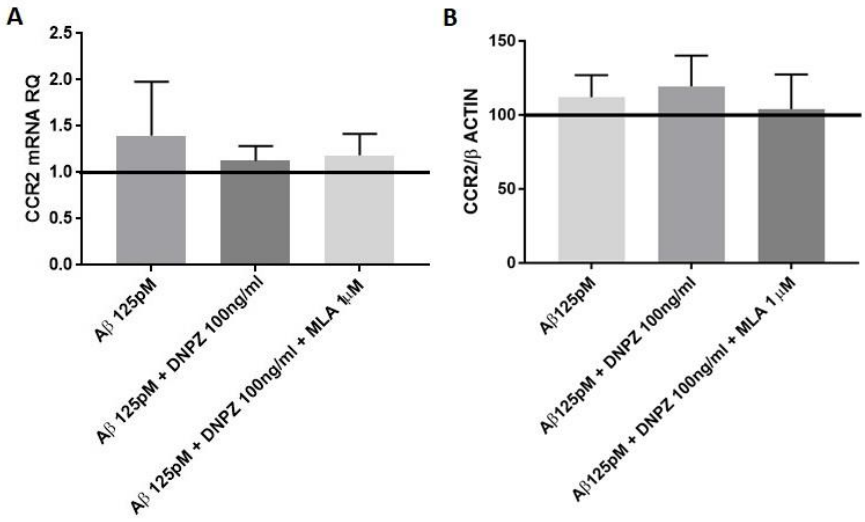


Figure 14: (A) CCR2 mRNA expression levels in U-937 cells do not change after treatment with Donepezil (n=4; p>0.05 at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD); basal (horizontal line) equals a CCR2 mRNA RQ of 1. **(B)** Similarly, CCR2 protein expression levels are not altered upon Donepezil's treatment (n=4; p>0.05 at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD); basal (horizontal line) equals a percentage CCR2/βACTIN ratio of 100.

- ***Co-ultraPEALut***

The anti-inflammatory effect of Co-ultraPEALut on monocytes migration has been evaluated on U-937 monocytic cell lines and human monocytes. Oligomeric Aβ42 125 pM was used as chemoattractant and, in addition, monocytes were stimulated with Co-ultraPEALut at different concentrations (100 nM, 500 nM, 1 µM and 10 µM for U-937; 1 µM and 500 nM for AD monocytes) already proven to be effective in experimental AD models [309]. Experiments on U-937 monocytic cell lines demonstrated that Co-ultraPEALut is able to prevent Aβ-induced chemotaxis in a concentration dependent manner, notwithstanding a slight increase in cell motility occurring at a concentration of 10 µM. In particular, Co-ultraPEALut 1 µM significantly inhibited Aβ-induced migration (Aβ 4.7 ± 3.5 vs. Aβ+Co-ultraPEALut 1 µM 2.1 ± 1.1; $p<0.05$ Aβ+Co-ultraPEALut 1 µM vs. Aβ). The results were confirmed on human monocytes from AD patients (Aβ 3.0 ± 1.7 vs. Aβ+Co-ultraPEALut 1 µM 1.4 ± 1.0; $p<0.05$ Aβ+Co-ultraPEALut 1 µM vs. Aβ) (**Figure 15**).

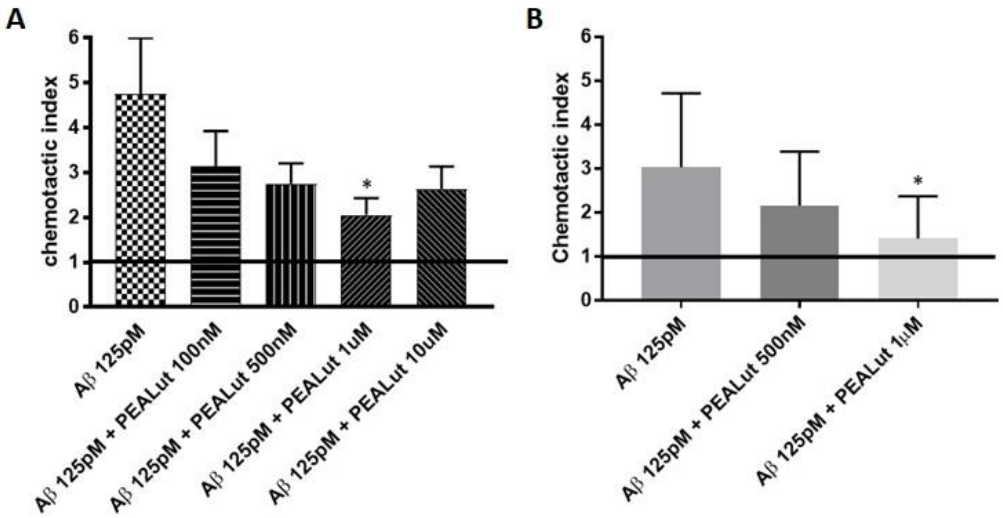


Figure 15: (A) Co-ultraPEALut prevents Aβ-induced chemotaxis in a concentration dependent manner in U-937 monocytic cell lines (n=8; $p<0.05$ Co-ultraPEALut 1 μM vs. Aβ; $p<0.002$ at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD) **(B)** and monocytes from AD patients (n=7; $p<0.05$ Aβ+Co-ultraPEALut 1μM vs. Aβ; $p<0.05$ at ANOVA, followed by Sidak's multiple comparisons test, mean ± SD). Basal (horizontal line) equals a chemotactic index of 1.

- **Co-ultraPEALut modulation of Aβ-induced CCR2 expression**

The effect of Co-ultraPEALut on CCR2 mRNA and protein expression levels has been evaluated in U-937 cells after stimulation with oligomeric Aβ42 125 pM, alone or in combination with Co-ultraPEALut at different concentrations (100 nM, 500 nM, 1 μM and 10 μM). Both Real-time PCR and Western blot analysis showed that Co-ultraPEALut is not able to alter CCR2 expression (**Figure 16**).

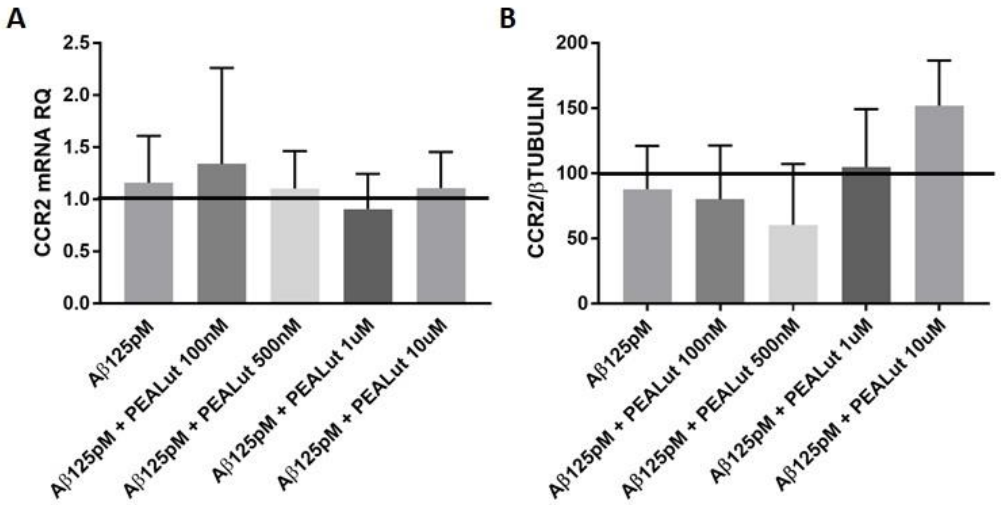


Figure 16: (A) CCR2 mRNA expression levels in U-937 cells do not change after treatment with Co-ultraPEALut (n=3; p>0.05 at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD); basal (horizontal line) equals a CCR2mRNA RQ of 1. **(B)** Co-ultraPEALut is not able to alter CCR2 protein expression (n=3; p>0.05 at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD); basal (horizontal line) equals a percentage CCR2/βTUBULIN ratio of 100.

- ***Anti-Aβ monoclonal antibodies***

The effect of Aβ sequestration by specific monoclonal antibodies on peripheral monocytes chemotactic properties has been tested on U-937 monocytic cell lines and human monocytes from AD patients exposed to either oligomeric Aβ42 125 pM or Aβ-mAb immunocomplexes. Anti-Aβ monoclonal antibodies, at a concentration comparable to that of NAb detected in the CSF of AD patients (25 ng/ml) [310,311], induce a significant reduction of Aβ-induced migration in monocytic cell lines (Aβ 3.0 ± 0.2 vs. IC 1.5 ± 0.2; p<0.001 IC vs. Aβ) and human monocytes from AD patients (Aβ 2.5 ± 1.4 vs. IC 1.7 ± 0.7; p<0.05 IC vs. Aβ) (**Figure 17**).

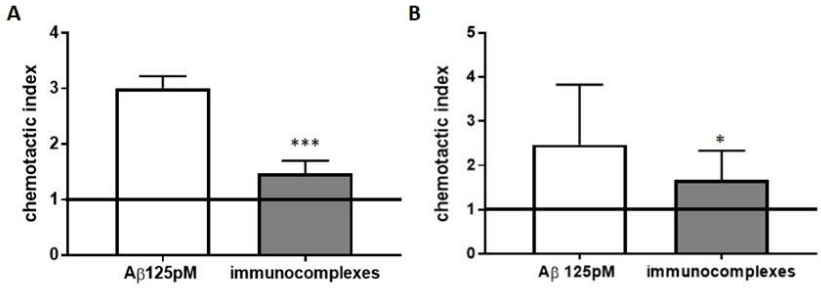


Figure 17: (A) Anti-Aβ monoclonal antibodies induce a significant reduction of Aβ-induced migration in U-937 monocytic cell lines (n=3; p<0.001 IC vs. Aβ; p<0.001 at ANOVA, followed by Tukey's multiple comparisons test; mean ± SD) **(B)** and human monocytes from AD patients (n=9; p<0.05 IC vs. Aβ; p<0.001 at ANOVA, followed by Tukey's multiple comparisons test; mean ± SD). Basal (horizontal line) equals a chemotactic index of 1.

- **Anti-Aβ antibodies modulation of Aβ-induced CCR2 expression**

The effect of Aβ sequestration by anti-Aβ mAb on CCR2 mRNA and protein expression levels has been evaluated in U-937 cells after stimulation with oligomeric Aβ42 (125 pM) or Aβ-mAb immunocomplexes. Both Real-time PCR and Western blot analysis showed that anti-Aβ monoclonal antibodies are not able to alter CCR2 mRNA and protein expression levels through Aβ sequestration (**Figure 18**).

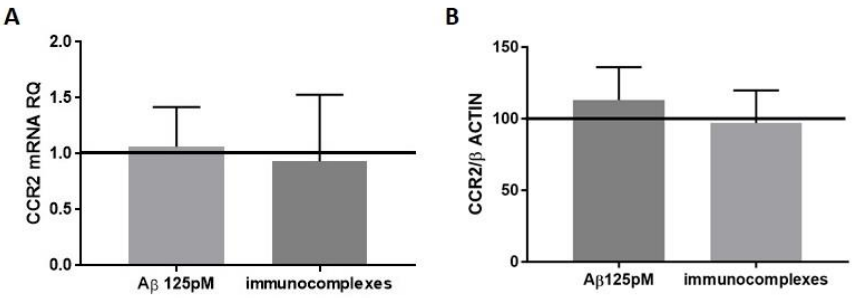


Figure 18: (A) Aβ sequestration by anti-Aβ mAb does not alter CCR2 mRNA expression level in U-937 cells (n=5; p>0.05 at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD); basal (horizontal line) equals a CCR2mRNA RQ of 1. **(B)** Similarly, CCR2 protein expression levels are not altered by anti-Aβ mAb sequestration of Aβ (n=4; p>0.05 at ANOVA, followed by Tukey's multiple comparisons test, mean ± SD); basal (horizontal line) equals a percentage CCR2/βACTIN ratio of 100.

C. Phagocytosis

6.C.1 Effect of Donepezil on Aβ internalization

With the intent of evaluating the putative effect of Donepezil treatment on Aβ internalization, phagocytosis assays were performed on monocytic cell lines differentiated into macrophages and human macrophages; moreover, the expression of TREM2 - the principal receptor involved in the regulation of phagocytosis in myeloid cells - was evaluated.

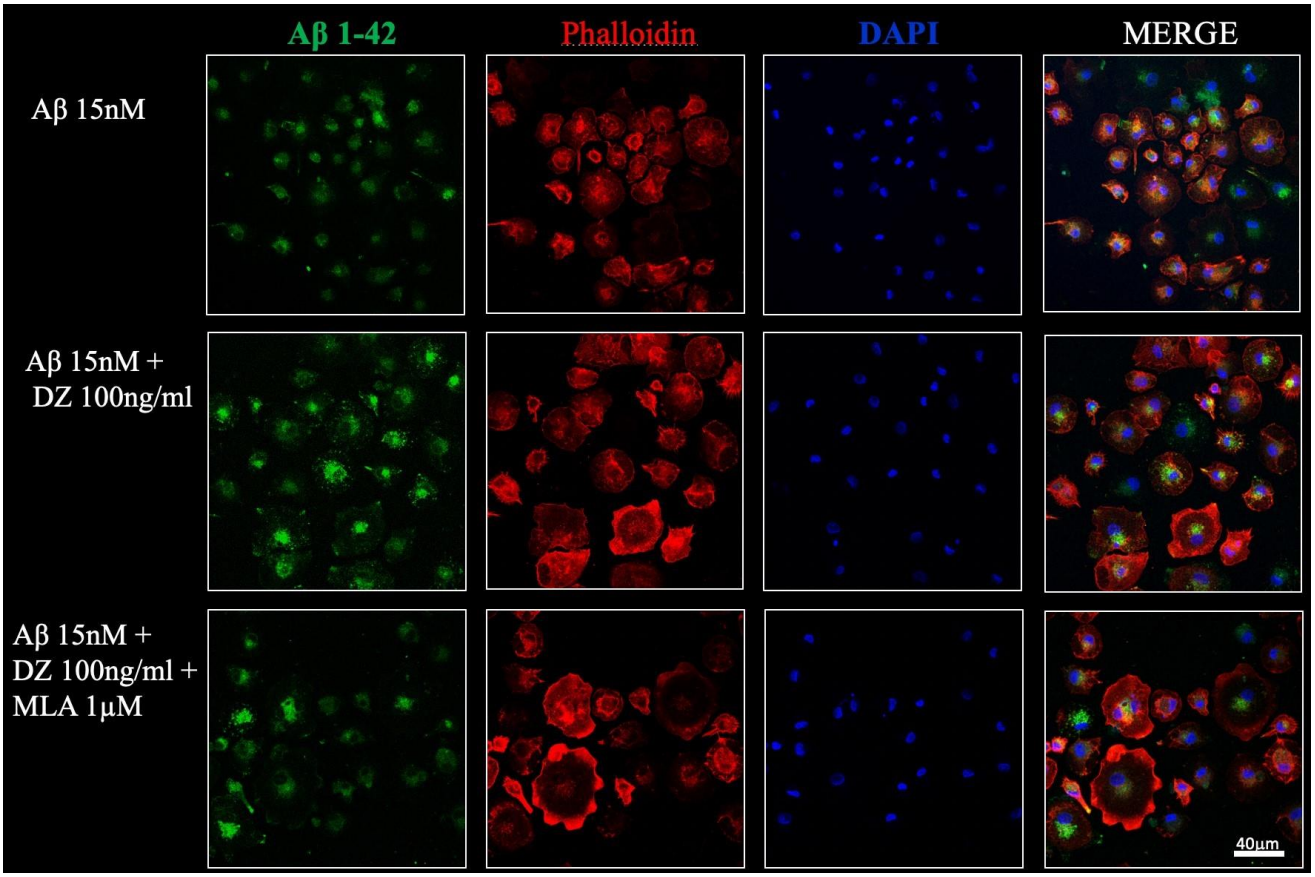
6.C.1.1 Phagocytosis assay

Fluorescence microscopy analysis was used to assess fluorescent Aβ internalization on both U-937 cells and human macrophages upon stimulation with Donepezil. For this purpose, fluorescent oligomeric Aβ42 was used at a concentration in the same order of magnitude as the concentration of the protein in the human brain of AD patients (15 nM) [312,313].

- **Phagocytosis in U-937 macrophages**

U-937 cells have been differentiated into macrophages and subsequently stimulated with fluorescent oligomeric Aβ42 15 nM, alone or in combination with Donepezil at different concentrations (100 ng/ml, 1 µg/ml and 50 µg/ml) and/or pre-treated with α7nAChR inhibitor MLA 1 µM. Results revealed that Donepezil treatment increased fluorescent Aβ internalization (Aβ+DNPZ 100 ng/ml 147.2 ± 4.3 vs. Aβ+DNPZ 1 µg/ml 123.5 ± 3.6 vs. Aβ+DNPZ 50 µg/ml 137.7 ± 2.7; p<0.001 Aβ vs. Aβ+DNPZ 100

ng/ml; p<0.01 Aβ vs. Aβ+DNPZ 1 µg/ml; p<0.001 Aβ vs. Aβ+DNPZ 50 µg/ml). The process appears to be α7nAChR-mediated, since the addition of nicotinic receptor inhibitor MLA to DNPZ 100 ng/ml treated cells restored internalization to basal levels (Aβ+DNPZ 100 ng/ml + MLA 126.9 ± 11.1; p<0.05 Aβ+DNPZ 100 ng/ml vs. Aβ+DNPZ 100 ng/ml + MLA) (**Figure 19**).



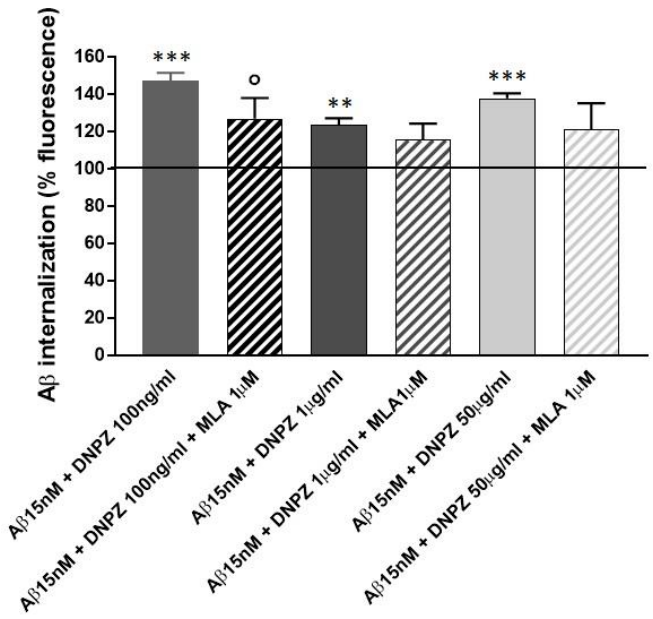


Figure 19: Representative images of fluorescence microscopy show Aβ in green (Fluor 488), nuclei in blue (DAPI) and cytoplasm in red (Phalloidin). Compared to basal condition, Donepezil treatment increases fluorescent Aβ internalization in U-937 macrophages (n=4; $p<0.001$ Aβ vs. Aβ+DNPZ 100 ng/ml; $p<0.01$ Aβ vs. Aβ+DNPZ 1 μg/ml; $p<0.001$ Aβ vs. Aβ+DNPZ 50 μg/ml; $p<0.001$ at ANOVA followed by Tukey's multiple comparisons test; mean ± SD). Moreover, α7nAChR inhibitor MLA is able to reduce Aβ internalization in DNPZ 100 ng/ml treated cells ($p<0.05$ Aβ+DNPZ 100 ng/ml vs. Aβ+DNPZ 100 ng/ml + MLA); basal (horizontal line) equals a fluorescent Aβ internalization of 100%.

Concurrently, Aβ42 Solid-Phase Enzyme Immunoassay was employed to confirm the results: the residual (not-internalized) fluorescent Aβ in the culture medium of macrophages treated with Donepezil 100 ng/ml and/or pre-treated with MLA was quantified, showing a significant reduction in Donepezil treated cells compared to baseline and MLA pre-treated cells (Aβ 5.5 ± 1.1 vs. Aβ+DNPZ 4.3 ± 0.7 vs. Aβ+DNPZ+MLA 5.0 ± 0.7; $p<0.001$ Aβ vs. Aβ+DNPZ; $p<0.01$ Aβ+DNPZ vs. Aβ+DNPZ+MLA) (**Figure 20**).

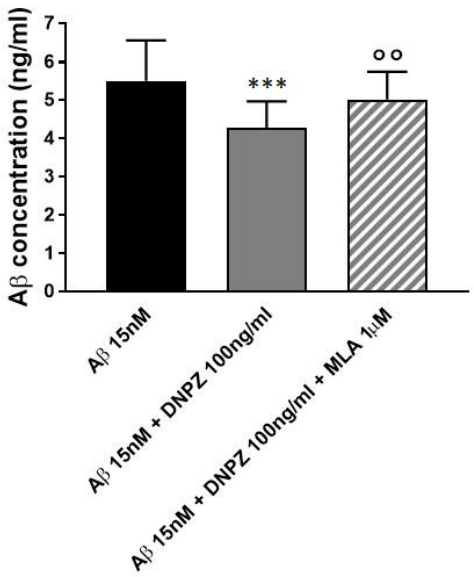
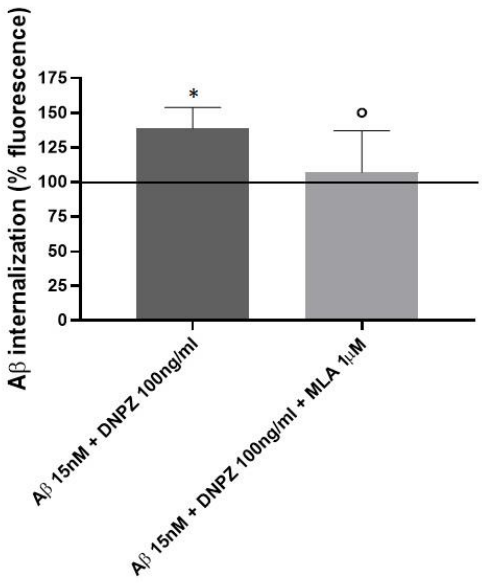


Figure 20: Fluorescent Aβ level decreases in the culture medium of Donepezil treated macrophages compared to baseline (n=4; $p<0.001$ Aβ vs. Aβ+DNPZ; $p<0.001$ at ANOVA followed by Tukey's multiple comparisons test; mean ± SD). On the other hand, fluorescent Aβ level is increased in the culture medium of MLA pre-treated cells ($p<0.01$ Aβ+DNPZ vs. Aβ+DNPZ+MLA).

- **Phagocytosis in human macrophages**

Monocytes from AD patients and healthy controls have been differentiated into macrophages and subsequently stimulated with fluorescent oligomeric Aβ42 15 nM, alone or in combination with Donepezil 100 ng/ml and/or pre-treated with MLA 1 μM. Fluorescence microscopy analysis revealed that Donepezil was able to increase Aβ internalization compared to baseline in macrophages from control subjects (Aβ+DNPZ 139.0 ± 15.0; $p<0.05$ Aβ vs. Aβ+DNPZ). Moreover, MLA reduced Aβ internalization in Donepezil-treated cells, restoring it to basal levels (Aβ+DNPZ+MLA 107.0 ± 30.1; $p<0.05$ Aβ+DNPZ vs. Aβ+DNPZ+MLA) (**Figure 21**).



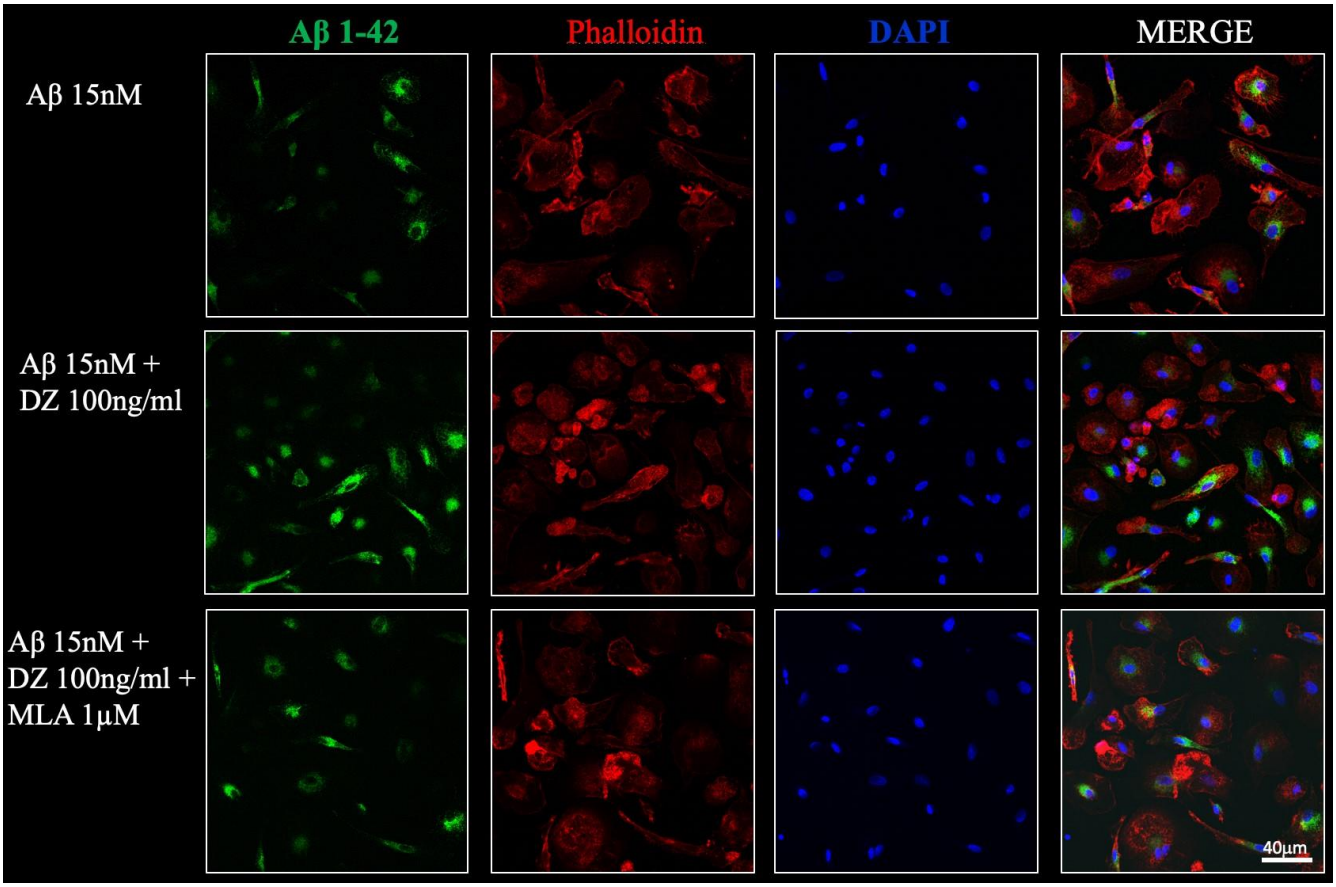


Figure 21: Donepezil increases fluorescent Aβ internalization in macrophages from healthy controls compared to baseline (n=4; $p<0.05$ Aβ vs. Aβ+DNPZ; $p<0.02$ at ANOVA followed by Bonferroni's multiple comparisons test; mean ± SD). MLA restores Aβ internalization in Donepezil-treated cells to basal levels ($p<0.05$ Aβ+DNPZ vs. Aβ+DNPZ+MLA); basal (horizontal line) equals a fluorescent Aβ internalization of 100%. Representative images of fluorescence microscopy show Aβ in green (Fluor 488), nuclei in blue (DAPI) and cytoplasm in red (Phalloidin).

On the other hand, no difference in terms of Aβ internalization have been observed in Donepezil-treated macrophages from AD patients compared to baseline (**Figure 22**).

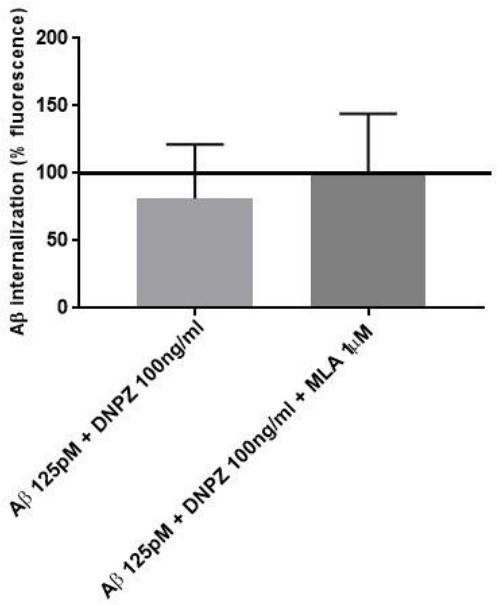


Figure 22: Donepezil has no effect on fluorescent Aβ internalization in human macrophages from AD patients (n=12; p>0.05 at ANOVA followed by Tukey's multiple comparisons test; mean ± SD); basal (horizontal line) equals a fluorescent Aβ internalization of 100%.

6.C.1.2 TREM2 expression

To verify if differences in phagocytic activity could be dependent on the modulation of TREM2 expression, Real-time PCR and Western blot were performed in either U-937 cells differentiated into macrophages and human macrophages.

- **TREM2 expression in U-937 macrophages**

TREM2 mRNA and protein expression were evaluated in U-937 macrophages upon stimulation with Donepezil, using Nicotine (established α7nAChR agonist) as positive control. In detail, U-937 cells were differentiated into macrophages and subsequently stimulated with oligomeric Aβ42 (125 pM), alone or in combination with either Donepezil (100 ng/ml) or Nicotine (50 μM), and/or pre-treated with MLA (1 μM). Real-time PCR showed a dramatic reduction in TREM2 expression compared to basal condition upon stimulation with Aβ (Aβ 0.1 ± 0.0; $p<0.001$ basal vs. Aβ) and a significant increase in response to

either Donepezil (Aβ+DNPZ 0.8 ± 0.3; p<0.01 Aβ vs. Aβ+DNPZ) or Nicotine treatment (Aβ+N 0.8 ± 0.3; p<0.01 Aβ vs. Aβ+N). As for MLA, this α7nAChR inhibitor had no significant effect on neither Donepezil nor Nicotine-mediated increase in TREM2 expression (**Figure 23**).

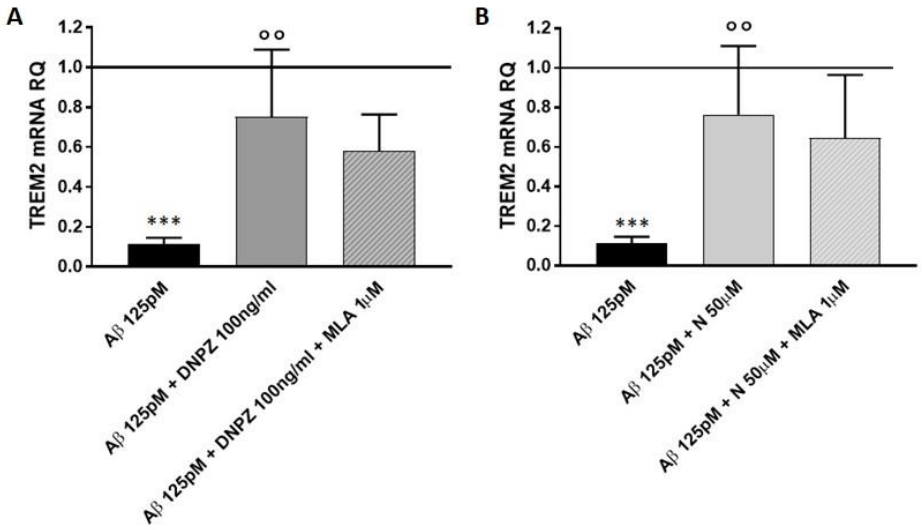


Figure 23: TREM2 mRNA expression in U-937 macrophages is reduced upon stimulation with Aβ (n=3; p<0.001 basal vs. Aβ). **(A)** Donepezil increases TREM2 expression in Aβ-treated cells (p<0.01 Aβ vs. Aβ+DNPZ; p<0.001 at ANOVA followed by Bonferroni's multiple comparisons test; mean ± SD). **(B)** Nicotine also increases TREM2 expression in Aβ-treated cells (p<0.01 Aβ vs. Aβ+N; p<0.001 at ANOVA followed by Bonferroni's multiple comparisons test; mean ± SD). MLA has no effect on Donepezil-mediated or Nicotine-mediated increase in TREM2 expression. Basal (horizontal line) equals a TREM2 mRNA RQ of 1.

Western blot analysis showed that Aβ stimulation was able to induce a significant reduction in TREM2 expression compared to basal condition (p<0.05 basal vs. Aβ), and that a significant increase in TREM2 expression in Aβ-treated cells is induced by both Donepezil (Aβ 58.8 ± 24.1 vs. Aβ+DNPZ 115.7 ± 20.4; p<0.01 Aβ vs. Aβ+DNPZ) and Nicotine (Aβ 47.6 ± 10.9 vs. Aβ+N 156.2 ± 56.5; p<0.01 Aβ vs. Aβ+N). In addition, MLA was able to lower TREM2 protein expression in both Donepezil-treated (Aβ+DNPZ+MLA 51.3 ± 27.1; p<0.01 Aβ+DNPZ vs. Aβ+DNPZ+MLA) and Nicotine-treated cells (Aβ+N+MLA 64.9 ± 19.8; p<0.01 Aβ+N vs. Aβ+N+MLA) (**Figure 24**).

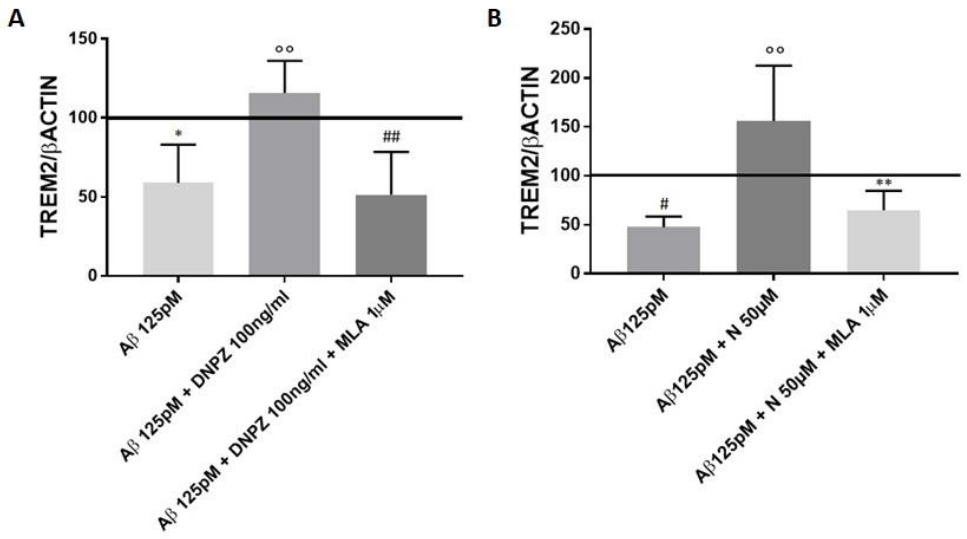


Figure 24: TREM2 protein expression in U-937 macrophages is reduced upon stimulation with Aβ (n=5; $p<0.05$ basal vs. Aβ; $p<0.01$ at ANOVA followed by Tukey's multiple comparisons test; mean ± SD). Donepezil **(A)** and Nicotine **(B)** increase TREM2 expression in Aβ-treated cells ($p<0.01$ Aβ vs. Aβ+DNPZ; $p<0.01$ Aβ vs. Aβ+N). MLA reduces Donepezil-mediated **(A)** and Nicotine-mediated **(B)** increase in TREM2 expression ($p<0.01$ Aβ+DNPZ vs. Aβ+DNPZ+MLA; $p<0.01$ Aβ+N vs. Aβ+N+MLA). Basal (horizontal line) equals a percentage TREM2/βACTIN ratio of 100.

- **TREM2 expression in human macrophages**

TREM2 mRNA expression has been quantified in monocytes from AD patients and controls differentiated into macrophages. Real-time PCR showed lower TREM2 mRNA expression in AD patients compared to healthy controls (AD 0.9 ± 0.6 vs. CTRL 1.2 ± 0.6; $p<0.05$) (**Figure 25**).

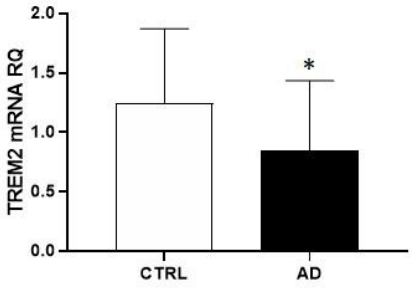


Figure 25: TREM2 mRNA expression is reduced in AD patients (n=48) compared to healthy controls (n=20; $p<0.05$ at unpaired two tailed t-test; mean ± SD).

In addition, TREM2 mRNA expression was evaluated in AD patients divided according to Donepezil treatment. Donepezil-treated patients exhibited increased TREM2 expression compared to untreated AD patients (DNPZ+ 1.4 ± 1.0 vs. DNPZ- 0.7 ± 0.4; p<0.01 DNPZ+ vs. DNPZ-) (**Figure 26**).

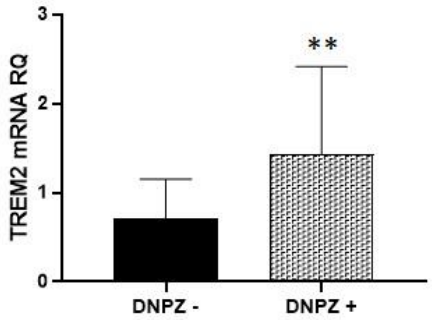


Figure 26: Donepezil-treated patients (n=28) exhibit increased TREM2 expression compared to untreated patients (n=20; p<0.01 at unpaired two tailed t-test; mean ± SD).

- **soluble TREM2 levels**

ELISA assay was performed to quantify the soluble form of TREM2 (sTREM2) - supposedly contributing to Aβ uptake and degradation - in plasma of AD patients and controls already tested for TREM2 mRNA expression. Results revealed no difference in terms of sTREM2 plasma levels between AD patients and controls (CTRL 37.4 ± 10.8 vs. AD 42.4 ± 21.2) (**Figure 27**).

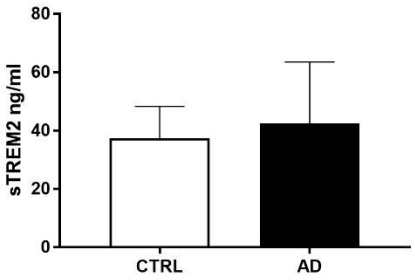


Figure 27: sTREM2 plasma levels do not differ between AD patients (n=48) and controls (n=20; p>0.05 unpaired two tailed t-test; mean ± SD).

Also, sTREM2 has been quantified in plasma of AD patients divided according to Donepezil treatment. ELISA showed increased sTREM2 expression in Donepezil-treated patients compared to untreated patients (DNPZ+ 47.8 ± 23.9 vs. DNPZ- 33.5 ± 9.5, p<0.01 DNPZ+ vs. DNPZ-) (**Figure 28**).

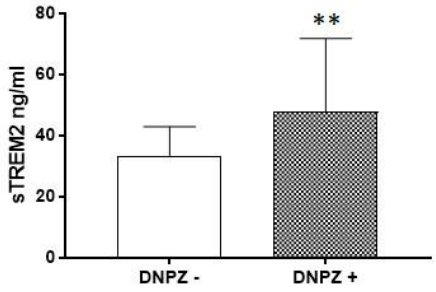


Figure 28: sTREM2 plasma levels are increased in Donepezil-treated patients (n=28) compared to untreated ones (n=20; $p<0.01$ at unpaired two tailed t-test; mean ± SD).

6.C.2 Effect of Co-ultraPEALut on Aβ internalization

To assess the putative effect of Co-ultraPEALut treatment on Aβ internalization, TREM2 mRNA and protein expression levels were assessed in U-937 monocytic cell lines differentiated into macrophages upon stimulation with Co-ultraPEALut at different concentrations (500 nM and 1 µM). Real-time PCR showed a significant reduction in TREM2 expression compared to basal condition upon stimulation with Aβ (Aβ 0.7 ± 0.01; $p<0.05$ basal vs. Aβ). In addition, Co-ultraPEALut 1 µM treatment has proved able to restore Aβ-mediate TREM2 reduction to basal levels (Aβ+Co-ultraPEALut 1 µM 1.0 ± 0.2; $p<0.05$ Aβ vs. Aβ+Co-ultraPEALut 1 µM) (**Figure 29**).

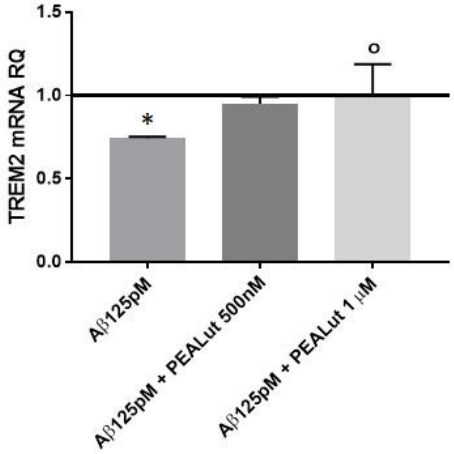


Figure 29: TREM2 mRNA expression is reduced upon stimulation with Aβ (n=4; $p<0.05$ at ANOVA followed by Sidak's multiple comparisons test; mean ± SD), and increases in cells treated with Co-ultraPEALut 1 µM ($p<0.05$ Aβ+Co-ultraPEALut 1 µM vs. Aβ). Basal (horizontal line) equals a TREM2 mRNA RQ of 1.

Western blot analysis showed a significant reduction in TREM2 protein expression - compared to basal - following stimulation with Aβ (Aβ 55.3 ± 6.3, p<0.001 basal vs. Aβ). On the other hand, a significant increase in the expression level was observed in cells treated with Co-ultraPEALut 500 nM and 1 µM, compared to those stimulated only with Aβ (Co-ultraPEALut 500 nM 137.0 ± 30.5 vs. Co-ultraPEALut 1 µM 115.0 ± 26.2; p<0.01 Aβ+Co-ultraPEALut 500 nM vs. Aβ; p<0.05 Aβ+Co-ultraPEALut 1 µM vs. Aβ) (**Figure 30**).

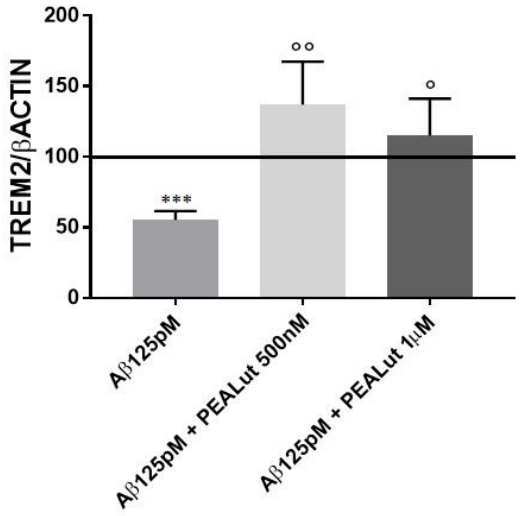


Figure 30: TREM2 protein expression is reduced upon stimulation with Aβ (n=3; p<0.001 Aβ vs. basal; p<0.01 at ANOVA followed by Tukey's multiple comparisons test; mean ± SD), and increases in cells treated with Co-ultraPEALut 500 nM and 1 µM (p<0.01 Aβ+Co-ultraPEALut 500 nM vs. Aβ; p<0.05 Aβ+Co-ultraPEALut 1 µM vs. Aβ). Basal (horizontal line) equals a percentage TREM2/βACTIN ratio of 100.

6.C.3 Effect of Anti-Aβ monoclonal antibodies on Aβ internalization

To determine the putative effect of anti-Aβ monoclonal antibodies on Aβ internalization, phagocytosis assay was performed on human macrophages. Moreover, the expression of TREM2 was evaluated.

6.C.3.1 Phagocytosis assay

Human monocytes from AD patients have been differentiated into macrophages and subsequently stimulated with fluorescent oligomeric Aβ42 15nM alone or with fluorescent Aβ-mAb immunocomplexes. Fluorescence microscopy analysis revealed that Aβ sequestration by anti-Aβ

monoclonal antibodies does not alter the phagocytic activity of human macrophages collected from AD patients (**Figure 31**).

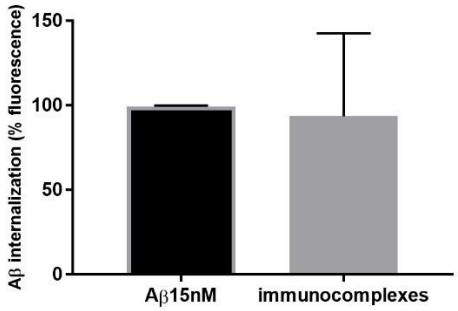


Figure 31: Aβ sequestration by anti-Aβ monoclonal antibodies does not alter the phagocytic activity of human macrophages collected from AD patients. (n=7; p>0.05 at paired two tailed t-test; mean ± SD).

6.C.3.2 TREM2 expression

The possible effect of anti-Aβ monoclonal antibodies on TREM2 mRNA expression levels in the context of phagocytosis was evaluated on U-937 monocytic cell lines differentiated into macrophages. Real-time PCR showed a significant reduction in TREM2 expression compared to basal after stimulation with Aβ (Aβ 0.7 ± 0.01; p<0.05 basal vs. Aβ), but failed to show any enhancement after Aβ sequestration by means of anti-Aβ mAb (**Figure 32**).

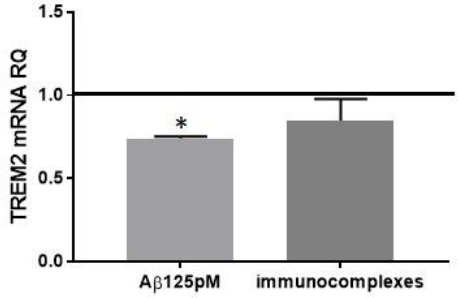


Figure 32: TREM2 mRNA expression is reduced upon stimulation with Aβ (n=3; p<0.05 basal vs. Aβ; p<0.05 at ANOVA followed by Sidak's multiple comparisons test; mean ± SD). Anti-Aβ mAb pre-treatment fails to restore TREM2 levels to basal conditions. Basal (horizontal line) equals a TREM2 mRNA RQ of 1.

D. DBI/TSPO system in A/A

6.D.1 DBI levels

The first step towards the elucidation of the putative role of the DBI/TSPO system in the pathogenesis of agitation/aggression behavioural symptoms in AD was the evaluation of DBI levels in serum and CSF of agitated/aggressive AD patients and their non-agitated/aggressive counterparts. DBI ELISA assay showed that both CSF (AA- 25.8 ± 13.0 vs. AA+ 24.0 ± 7.1) and serum (AA- 39.1 ± 14.9 vs. AA+ 41.2 ± 18.8) DBI levels fail to segregate with the A/A phenotype (**Figure 33**).

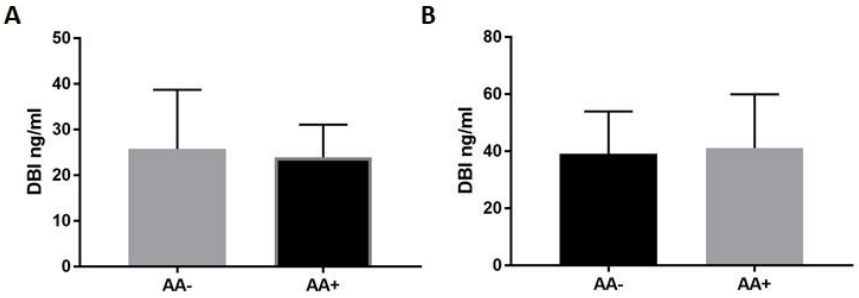


Figure 33: (A) CSF levels of DBI do not show any difference between AA+ (n=6) and AA- patients (n=6; p>0.05 at unpaired two tailed t-test; mean ± SD). **(B)** Serum DBI levels do not differ between AA+ (n=20) and AA- patients (n=34; p>0.05 at unpaired two tailed t-test; mean ± SD).

6.D.2 TSPO expression

In light of its role as classical receptor for DBI, TSPO mRNA and protein expression was assessed in PBMC from AD patients after A/A dichotomization. A modest decrease of TSPO levels in AA+ patients compared to AA- was observed in terms of both mRNA (AA+ 1.4 ± 0.7 vs. AA- 1.7 ± 0.8; p<0.05 AA+ vs. AA-) and protein expression (AA- 1.7 ± 0.3 vs. AA+ 0.8 ± 0.2; p<0.05 AA+ vs. AA-) (**Figure 34**).

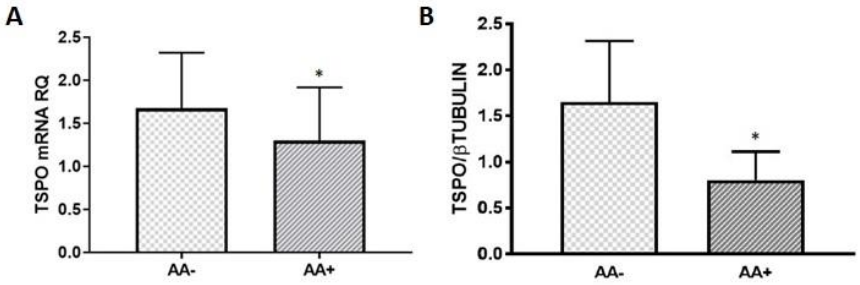


Figure 34: (A) TSPO mRNA level is slightly decreased in AA+ patients (n=24) compared to AA- (n=36; $p<0.05$ at unpaired two tailed t-test; mean ± SD). **(B)** Likewise, TSPO protein expression was reduced in AA+ patients (n=10) compared to AA- (n=10; $p<0.05$ at unpaired two tailed t-test; mean ± SD).

6.D.3 Peripheral monocyte chemotaxis

Taking into account the differences in terms of TSPO expression between AA+ and AA- patients, monocytes migration was quantified through Boyden chamber assay using oligomeric Aβ42 125 pM as chemotactic stimulus. No difference in chemotactic behavior was observed between the two groups (AA- 3.5 ± 0.7 vs. AA+ 3.5 ± 0.5) (**Figure 35**).

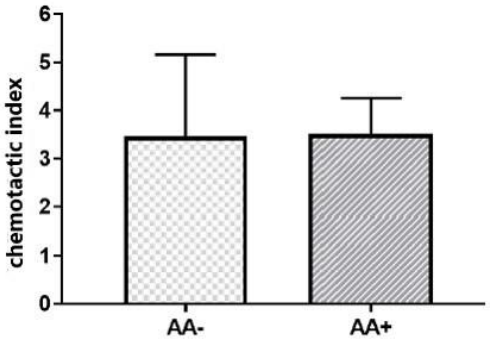


Figure 35: Monocytes from AA+ (n=3) and AA- patients (n=5) do not show any difference in terms of chemotactic activity ($p>0.05$ at unpaired two tailed t-test; mean ± SD)

Chapter 7
Discussion

The overall goal of this PhD project was to shed light on the contribution of peripheral monocytes to neuroinflammation, a central process in the pathogenesis of Alzheimer's disease and Behavioral and Psychological Symptoms of Dementia. To do so, the mechanisms underlying peripheral monocytes recruitment in the AD brain and their contribution to Aβ clearance were investigated. As a matter of fact, peripheral monocytes have been demonstrated to infiltrate the brain parenchyma of AD patients and associate with regions of increased Aβ deposition [176]. However, their contribution to AD pathogenesis is still controversial: one hypothesis is that, upon recognition of Aβ peptides, infiltrating monocytes differentiate into macrophages and contribute to the clearance of Aβ [173]; although, such conclusion is challenged by studies showing that infiltrating monocytes exhibit reduced differentiation into macrophages, have limited phagocytic activity [178] and contribute to fuel the chronic neuroinflammatory state [314].

In the first part of this project, the ability of Aβ42 - the principal protein accumulated in the AD brain - to promote peripheral monocytes chemotaxis was evaluated. The hypothesis was that the presence of Aβ aggregates could be enough by itself to influence the movement of peripheral immune cells into the AD brain. As a matter of fact, soluble Aβ was already proven to be a powerful chemotactic stimulus both in vitro blood-brain barrier models and in AD transgenic mice [156].

In our study, time-lapse microscopy results showed a clear tendency of THP-1 cells to migrate towards Aβ42 to the same extent as when they are exposed to MCP-1, the most effective chemotactic factor for monocytes. Boyden chambers experiments confirmed these results on human monocytes from elderly controls, showing an increased migration rate following exposure to Aβ42, even though in this case the effect resulted less evident respect to MCP-1 stimulated monocytes. Finally, the experiments suggested that monocytes obtained from AD patients are more prone to Aβ-induced chemotaxis compared to those from matched healthy controls. These results are in line with data from Zhang et al. [315], describing an increased ability of monocytes isolated from AD patients to cross a model of endothelial barrier upon Aβ

stimulation. However, compared to Zhang's study - in which Aβ42 was used at nanomolar concentrations, our work demonstrates the ability of monocytes to migrate toward Aβ42 at picomolar concentrations (125 pM), reflecting its levels in pathophysiological conditions.

The mechanisms underlying monocytes chemotactic response to Aβ42 stimulation were then investigated. In light of its leading role in the recruitment and accumulation of immune cells at the level of senile plaques [166,316], the CCR2/CCL2 signaling was the first to be explored. CCR2, the surface receptor for MCP-1 (or CCL2), is mainly expressed on the surface of monocytes [299], and recent evidences support the hypothesis that its downregulation in monocytes from AD patients [317] can contribute to Aβ accumulation and cognitive decline [318]. Since Aβ-induced upregulation of its ligand CCL2 has been demonstrated in microglia, astrocytes and human monocytes [168,169], the possibility that the same mechanism could also contribute to CCR2 modulation was taken into consideration. However, our results show that exposure to Aβ42 was not able to modify mRNA and protein levels of CCR2 in monocytic cell lines, and no difference in terms of CCR2 mRNA expression was detected in PBMC from AD patients compared to controls. Additional investigations would be required to determine whether is CCR2 cellular distribution - rather than its expression - to be affected by Aβ42 stimulation. As a matter of fact, the biological function of a receptor of this sort is intimately linked to its membrane localization, and the existence of an interdependence between chemotactic activity and CCR2 targeting to the plasma membrane of monocytes is highly plausible.

Parallel investigations focused on the putative role of TSPO, a transmembrane protein originally discovered as a peripheral binding site for benzodiazepines [225,226]. Although TSPO is primarily located on the outer mitochondrial membrane, it has also been identified on the plasma membrane of various cell types, including monocytes [174]. Endogenous ligands of TSPO have been shown to induce chemotaxis in peripheral immune cells [256,257], and various ex vivo human studies have demonstrated the ability of different exogenous ligands to influence the process in patients with generalized anxiety disorder [175,300].

To asses the contribution of TSPO to the regulation of Aβ-induced chemotaxis, the influence of different selective ligands, emerged as chemotactic modulators for peripheral monocytes, was evaluated: our results show that TSPO agonists Emapunil and Ro5-4864 were able to induce an increase of chemotactic properties in either monocytic cell lines and human monocytes from control subjects and AD patients. On the other hand, TSPO selective inhibitor PK11195 didn’t alter monocytic chemotactic behaviour

respect to basal conditions. Rather, it partially reverted Aβ-induced chemotaxis in a concentration dependent manner, suggesting that Aβ42 and PK11195 could be competing for the very same binding sites on the TSPO receptor. Taken together, these findings point towards an involvement of TSPO in the chemotaxis of peripheral monocytes in AD.

Our first hypothesis was that differences in monocyte chemotactic behavior could be dependent on the modulation of TSPO expression levels; however, neither exposure to Aβ42 nor to TSPO selective ligands were able to affect TSPO mRNA or protein abundance, that remained similar to basal conditions. As considered for CCR2, additional investigations should determine if TSPO cellular distribution could be affected by Aβ42 stimulation.

Subsequently, a possible correlation between monocyte chemotactic modulation and rs6971 TSPO polymorphism was explored. As a matter of fact, an alanine-to-threonine polymorphism (rs6971) in a region of TSPO that is highly conserved, results in a conformational change affecting its interaction with a variety of ligands for which affinity had already been demonstrated: the presence of the predominant form (alanine) is associated with high-affinity binding, while the polymorphism (threonine) shows reduced binding affinity [292]. Our idea was that a reduced affinity binding could be associated with decreased migration rate. Unfortunately, the evaluation of Aβ-induced chemotaxis in monocytes from AD patients characterized by different haplotypes didn't show any meaningful difference. In addition, no distinction was observed in terms of TSPO mRNA expression in PBMC from AD patients divided basing on their haplotype.

Concluding step in this first part of this study was the evaluation of the putative impact on Aβ-induced monocyte migration of some pharmacological and non-pharmacological compounds, currently employed/under development for the treatment of AD, with the potential to modulate neuroinflammation from the periphery by targeting cytokine/chemokine production, monocyte chemotaxis and Aβ phagocytosis. The assumption is that, in the current situation, therapies administered to patients are just symptomatic, thus unable to block the pathophysiological events leading to full-blown AD. Differently, it would be ideal to make use of the so-called "disease-modifying therapies" - treatments able to act on the cause and evolution of the disease by interfering with its pathogenesis and counteract or delay the appearance of symptoms [107].

Despite not being recognized yet as a disease-modifying medication, acetylcholinesterase inhibitor Donepezil - belonging to the first class of FDA approved drugs for mild to moderate AD treatment - has

proven effective in modulating the immune response in the specific context of AD by favoring a general shift toward an anti-inflammatory environment [109,224]. Its action seems to be mediated by the α7 nicotinic acetylcholine receptor expressed on monocytes/macrophages [221,222] and crucial player in the cholinergic anti-inflammatory pathway - a pathway activated in the CNS to monitor and regulate the release of pro-inflammatory cytokines from peripheral monocytes in real time [191]. Donepezil can act indirectly by either increasing the expression level of the α7nAChR [221] or prolonging the effect of ACh, and directly as noncompetitive agonist by activating the receptor through interaction with a different binding site from that of ACh [222].

In light of previous studies, demonstrating the ability of Nicotine - one of the principal agonists of α7nAChRs - to act on peripheral monocytes to restrain their chemotaxis [219], our assumption was that Donepezil as well could be able to modulate chemotaxis either as a direct agonist of the receptor or by potentiating the response evoked by its classical agonist ACh. Indeed, Boyden chambers experiments performed on human monocytes from AD patients and control subjects demonstrated the ability of the treatment to inhibit Aβ-induced migration. However, the process did not appear to be mediated by the α7nAChR, since no inhibition was observed by means of its high affinity inhibitor MLA [196]. In search for another receptor possibly involved in Donepezil-mediated modulation of Aβ-induced chemotaxis, our choice fell on CCR2. However, no difference in terms of CCR2 mRNA and protein expression levels were detected in monocytic cell lines upon stimulation with the compound.

In the future, it could be interesting to assess the possible involvement of TSPO in the process, by evaluating its expression and cellular distribution in response to Donepezil treatment. Moreover, it may be intriguing to explore Donepezil effect on α7nAChR-mediated inhibition of pro-migratory proteins, another mechanism putatively involved in the modulation of peripheral monocytes chemotaxis [319].

Even though pharmacological treatments are still the gold standard for the treatment of AD, the attention has recently converged on the potential neuroprotective role of natural compounds, able to attenuate or suppress inflammation. Co-ultraPEALut, is a co-ultramicronized nutraceutical constituted by the association of N-palmitoylethanolamine (PEA) and the antioxidant flavonoid luteolin, showing anti-inflammatory and antioxidant properties [125]. In experimental models of AD, published findings have demonstrated the beneficial effect of Co-ultraPEALut treatment as a result of its anti-inflammatory and neuroprotective action [126,127]; however, to the best of our knowledge, there are no reports of its effect over Aβ-induced recruitment of immune cells. Previous studies on cellular migration conducted separately on PEA and luteolin have produced conflicting results, showing inhibition of chemotaxis on

some cases [320,321], and increased migration on some other [322]. Insight on the combined effect of these two molecules on the chemotactic process are still lacking.

Here we show that Co-ultraPEALut prevented Aβ-induced chemotaxis in a concentration dependent manner in Boyden chambers experiments performed on monocytic cell lines and human monocytes from AD patients. Our results are in alignment with the conclusion drawn from the literature that this compound could represent a useful therapeutic agent with the potential to counteract neuroinflammation in AD. Subsequent studies investigating the mechanism underlying Co-ultraPEALut-mediated inhibition of Aβ-induced migration focused on CCR2, but failed to show any difference in terms of mRNA and protein expression levels after treatment with the nutraceutical. To conclude, just as it is for Donepezil, Co-ultraPEALut is probably able to favour an anti-inflammatory environment that acts by reducing monocyte migration. However, further studies are needed to understand the mechanisms involved in this process.

The anti-Aβ monoclonal antibody Aducanumab was the first ever disease-modifying medication approved by the FDA to treat patients with MCI or mild dementia stage of AD [114]. The idea that immunoglobulin preparations could represent an effective treatment for AD came from the observation of naturally occurring anti-Aβ auto-antibodies in the brain of AD patients, able to bind Aβ in the brain and promote its clearance [323]. A pathological reduction of these anti-Aβ NAbs is well known to take place during the course of AD [188,189] and, therefore, the main idea behind immunotherapy was to increase the levels of such specific immunoglobulins able to exert a protective action. Anti-Aβ antibodies - may they be naturally occurring or pharmaceutical products - are able to exert their mechanism of action by binding Aβ in the brain to promote its clearance [184], or by sequestering the protein in the peripheral blood to lower its free level [186]. Accordingly, the possibility that Aβ sequestration in the AD brain by means of specific immunoglobulins might reduce its chemotactic power towards peripheral monocytes was considered an interesting line of research.

Our introductory investigation on the subject focused on the effect on chemotaxis of Aβ sequestration by monoclonal antibodies at a concentration comparable to that of NAb detected in the CSF of AD patients [310,311]. Boyden chambers experiments performed on monocytic cell lines and human monocytes from AD patients showed that such immunoglobulins were actually able to induce a significant decrease in Aβ-induced migration. However, subsequent Real-time PCR and Western blot analysis invalidated the hypothesis that CCR2 expression could be downregulated by Aβ sequestration and play a role in the modulation of the process.

In the second part of this project, the phagocytic aptitude of peripheral macrophages in the context of AD pathology was determined by evaluating the expression of TREM2 and the circulating level of its soluble form (sTREM2).

TREM2 is one of the principal transmembrane receptors involved in the regulation of phagocytosis in myeloid cells [301]. Interestingly, it can undergo proteolytic cleavage and be released as a soluble form (sTREM2) possessing many functional aspects resembling those of the full-length membrane-bound protein [302]. In the AD brain, TREM2 seems to be overexpressed by amyloid-associated microglia and peripherally recruited monocytes/macrophages [324-327] where, upon binding oligomeric Aβ [328], it mediates their recruitment and accumulation around plaques [325,329], as well as amyloid uptake and degradation [330,331]. Nonetheless, such perspective is challenged by claims of reduced phagocytic activity ascribable to infiltrating monocytes/macrophages [178].

Contrary to the general consensus [327,332,333], here we show a significant reduction in TREM2 expression in lymphomonocytes from AD patients compared to healthy controls, putatively attributable to Aβ stimulation (as confirmed by experiments set up on monocytic cell lines). On the other hand, our results agree with the previous literature in terms of sTREM2 expression, showing no significant difference in plasma levels between AD patients and healthy controls [334,335]. Our hypothesis - in line with previous reports showing inhibition of TREM2 expression by means of LPS [336] – is that Aβ acts as a legit pro-inflammatory mediator, whose stimulation leads to deficiency of the receptor in peripheral macrophages. Of note, evidences from in vitro studies support the role of TREM2 in modulating immune cells polarization [337], with reduced M2 (pro-phagocytic) activation associated with downregulated receptor expression [338]. Accordingly, we came to believe that TREM2 deficiency in lympomonocytes from AD patients could be suggestive of a reduced ability to internalize Aβ.

Interestingly, we demonstrated that Donepezil treatment is able to produce a significant increase in TREM2 mRNA and protein expression in Aβ-treated macrophages, restoring it to basal levels, to the same extent as Nicotine, used as positive control. Accordingly, AD patients treated with Donepezil showed increased TREM2 expression in their lympomonocytes compared to untreated ones, together with an upregulated level of its soluble form (sTREM2) in their plasma. Of note, the increase in TREM2 expression appeared to be dependent on the activation of the α7nAChR, as confirmed by MLA-mediated inhibition of the process.

Concurrently, we were able to observe a substantial increase in TREM2 mRNA and protein expression in Aβ-treated macrophages by means of Co-ultraPEALut.
These data are perfectly in line with our preliminary hypothesis, wanting TREM2 expression to be the result of the surrounding milieu: more specifically, Donepezil (in virtue of its multifaceted interaction with the α7nAChR) and Co-ultraPEALut could promote a shift toward an anti-inflammatory environment, thereby favoring TREM2 upregulation.

Previous studies have clearly demonstrated that the presence of TREM2 on myeloid cells plays a crucial role in Aβ clearance, to the point that impaired phagocytosis is reported following loss of this receptor [330,331]. Therefore, our subsequent investigation aimed at assessing the impact of pharmacologically-induced TREM2 upregulation on the phagocytic activity of peripheral macrophages. In particular, we focused our attention on the specific effect of Donepezil.
According to the current literature, wanting α7nAChRs as high affinity binders for oligomeric Aβ42 [206] - with the potential to mediate its effective removal [207] - and AChEI as direct modulators of this process [215], we demonstrated that Donepezil is able to increase the internalization of fluorescent Aβ
in both cell lines and human macrophages from healthy controls. The data were confirmed by specific Aβ42 ELISA, showing diminished residual (not-internalized) fluorescent Aβ in the culture medium of macrophages treated with Donepezil compared to baseline. Noteworthy, the process seemed to be mediated by the α7nAChR, as confirmed by MLA-mediated inhibition of phagocytosis. Per contra, Donepezil failed to show any effect on Aβ clearance in human macrophages from AD patients.
Different hypothesis were conjectured to justify these results. For one, we can speculate that the reduced ability of Donepezil to influence phagocytosis could be the result of the pre-existing alterations of the cholinergic system in patients wherefrom the peripheral monocytes/macrophages were collected. As a matter of fact, one of the major features of AD is the reduction of α7nAChR levels in disease-relevant brain regions [212] as a result of degeneration of cholinergic neurons [57]. Under other conditions, the cause may be attributable to desensitization of the α7nAChRs triggered by chronic exposure to its agonists [339] (in this specific case, by prolonged exposure of AD patients to AChEI therapy) and resulting in its inactivation. Moreover, we reasoned that it could be Aβ itself to cause α7nAChR desensitization: previous literature has indeed reported that chronic exposure to Aβ42 at nanomolar concentrations, accumulating in the brain throughout AD progression, can lead to α7nAChR inactivation - putatively through a desensitization mechanism - resulting in dysregulation of normal receptor functioning [209] and enhanced receptor-peptide complex internalization [210,211]. Finally, Donepezil

failure to modulate phagocytosis of Aβ in human macrophages could be the result of a genetic predisposition of the patients wherefrom they were collected. Partial duplication of the α7nAChR gene [340], described in both human brain and periphery [341,342], is indeed responsible for inadequate translation of the resulting protein that acts as a dominant negative regulator of wild-type receptor function [343,344]. In particular, dup-α7nAChRs were shown to mediate deficient Aβ uptake in previous cellular studies [345]. Of note, this last hypothesis finds support in results from preliminary investigations conducted by our research group (data not shown), demonstrating that dup-α7nAChR expression is higher in AD patients unresponsive to AChEI treatment [346].

The outcome of preceding experiments, demonstrating the ability of Aβ to act as pro-inflammatory mediator favoring the downregulation of TREM2 in peripheral macrophages, drove our curiosity to evaluate if Aβ sequestration by means of specific monoclonal antibodies could have a counteractive effect (anti-inflammatory) and the potential to increase the expression of the receptor. However, Real time PCR analysis failed to show any enhancement in TREM2 expression after Aβ sequestration by means of anti-Aβ mAb. Of note, those antibodies were tested for our purposes at low concentrations, comparable to those of NAbs detected in the CSF of AD patients; therefore, it could be inferred that their level was inadequate to counterbalance the level of Aβ detected in the brain parenchyma of AD patients and induce a measurable reduction of its pro-inflammatory effect.

Subsequent investigations were carried out with the intent to assess the contribution of Aβ sequestration to peripheral macrophages-mediated phagocytosis. We proceeded from the well established evidence that a generic antibody is able to trigger internalization of its target protein once its constant portion is recognized by Fc receptors expressed on the plasma membrane of phagocytic cells [347]. Anti-Aβ antibodies, in particular, have been demonstrated to promote the FcR-mediated internalization of their specific target by microglia [348]. Nonetheless, in line with the notion that defective Aβ clearance from the brain is a major contributing factor to the pathophysiology of AD [39,40,137], the data here presented demonstrated that pathologically low anti-Aβ mAb intrathecal levels are not sufficient to drive phagocytosis of fluorescent Aβ in peripheral macrophages collected from AD patients. Interestingly, our results completely harmonize with those form Xiang et al. [305], which demonstrated that TREM2 deficiency interferes with the uptake efficacy of antibody-bound Aβ by phagocytic cells.

Our plan for the future is to verify if the increase of specific anti-Aβ immunoglobulins concentration in the brain of AD patient will deliver the expected protective effect in terms of TREM2 expression and plaque clearance. We can envisage different scenarios: on one hand, we can reproduce our experiments

by employing anti-Aβ mAb at a concentration comparable to that of Aducanumab in the brain after systemic administration [116]; on the other, we can aim at restoring the physiological concentration of anti-Aβ NAbs in the CSF, to assess if that's enough to positively impact TREM2 expression and phagocytosis. To this end, Donepezil treatment - in virtue of its ability to induce anti-Aβ42 immunoglobulins production by B lymphocytes [109,224] - would precisely serve our purpose.

In the last part of this project, we explored the potential implication of neuroinflammation in the agitation/aggression cluster of BPSD.

BPSD are a wide variety of disturbances usually manifesting during the course of AD [75,286]; agitation and aggression, in particular, occur in approximately 20% of AD patients, significantly affecting their quality of life and that of their caregivers [349]. The rationale behind our idea to explore the role of neuroinflammation in agitation/aggression was its clinical resemblance with another condition characterized by neuroinflammation as key pathological processes: hyperkinetic delirium [287,350], an extreme form of sickness behavior characterized by psychomotor alterations. Interestingly, delirium patients have been found to show elevated DBI serum levels, especially in the hyperkinetic subtype [276]. Since DBI levels are also increased in serum and CSF of AD patients [275,276], we hypothesized that the overstimulation of the DBI/TSPO system could be involved in the neuroinflammatory process underlying the genesis of agitation/aggression behavioural disturbances. In particular, in respect of the central aim of this study, we conjectured about the specific role of peripheral monocytes in the process, with a special focus on TSPO-mediated chemotaxis [175,256].

The first step towards the elucidation of the putative role of the DBI/TSPO system in the pathogenesis of A/A behavioural symptoms in AD was the evaluation of DBI levels in serum and CSF of agitated/aggressive AD patients and their non-agitated/aggressive counterparts. However, our results failed to show the expected increase in DBI to segregate with the A/A phenotype.

In light of its role as classical receptor for DBI, we concurrently analyzed TSPO expression in lymphomonocytes from AD patients dichotomized based on the presence/absence of the A/A symptomatology, witnessing a slight decrease in terms of mRNA and protein levels in agitated/aggressive patients. Here as well, the results were different from we had envisioned: an increased expression of the TSPO gene in peripheral monocytes ascribable to neuroinflammation [274].

Therefore, we came to the conclusion that the DBI/TSPO system may not be involved in A/A pathogenesis, as further confirmed by the inconclusive results emerged from Boyden chamber experiments.

There are, however, several elements to consider: first of all, these are only preliminary data and further research in a large number of A/A patients would be required to confirm the concluding results of this introductory study; also, the fact that our observations do not apply to the agitation/aggression cluster does not exclude the possibility of them being consistent with other symptom profiles in the BPSD spectrum. Finally, the role of the DBI/TSPO system in BPSD should be also considered in light of its involvement in the biosynthesis of neurosteroids and their downstream action on the GABAergic transmission [252].

Chapter 8
Conclusion

Neuroinflammation is an inflammatory response originated in the CNS that has been described in AD since the first characterization of the disorder [1], and that is now well recognized as a central feature in its pathogenesis [2]. The main trigger for neuroinflammatory responses in AD is the pathological accumulation of Aβ and NFT in the brain, and the principal players are represented by resident glial cells [3], whose activation leads to a defensive response aimed at promoting Aβ clearance [4]. However, it is now widely accepted that the peripheral immune system is also able to contribute to the neuroinflammatory process taking place in AD. In particular, peripheral monocytes have been demonstrated to infiltrate the brain parenchyma of AD patients and associate with regions of increased Aβ deposition [5]. Still, their contribution to AD pathogenesis remains controversial.

The main purpose of this PhD project was to investigate the mechanisms underlying peripheral monocytes recruitment in the AD brain and to understand their contribution to Aβ clearance.

Throughout our study we were able to demonstrate that Aβ42 - the principal protein accumulated in the AD brain - promotes peripheral monocytes migration, and that monocytes obtained from AD patients are more prone to Aβ-induced chemotaxis compared to those from matched healthy controls. However, the mechanisms underlying monocytes chemotactic response to Aβ42 stimulation are not yet completely elucidated. We observed that exposure to Aβ42 was not able to modify CCR2 expression in monocytic cell lines, and no difference in CCR2 abundance was detected in lymphomonocytes from AD patients compared to controls. Moreover, even though we were able to establish the involvement of TSPO in the chemotaxis of peripheral monocytes in AD, the modulation of its expression levels could not be held accountable for the increased chemotactic response towards Aβ42. Additional investigations would be required to determine whether is CCR2 and TSPO cellular distribution - rather than their expression - to be affected by Aβ42 stimulation.

Moreover, we determined the phagocytic aptitude of peripheral macrophages in the context of AD pathology by evaluating the expression of TREM2 and the circulating level of its soluble form (sTREM2). Despite finding no difference in sTREM2 plasma levels between AD patients and healthy controls, we showed a significant reduction in TREM2 expression in lymphomonocytes, putatively attributable to Aβ stimulation. In line with current thinking [6], we came to the assumption that macrophages from AD patients could be characterized by a reduced phagocytic activity.

Secondary goal was to evaluate the disease-modifying potential [7] of some pharmacological and non-pharmacological compounds currently employed/under development for the treatment of AD. In compliance with the principal aim of this study, our focus was on evaluating their ability to limit the extent of peripheral monocytes infiltrating in the AD brain, in order to reduce the chronic neuroinflammatory state they contribute to create [8]; on the other hand, we aimed at assessing their ability to promote the differentiation into functional macrophages of those monocytes that were able to access the brain parenchyma, thereby giving a positive contribution to Aβ clearance.

First of all, we showed that acetylcholinesterase inhibitor Donepezil - belonging to the first class of FDA approved drugs for mild to moderate AD treatment [9] - is able to inhibit Aβ-induced monocytes migration. However, the process did not appear to be influenced neither by the activation of the α7nAChR (principal mediator of its immunomodulatory action [10]) nor by the modulation of CCR2 expression levels.
Interestingly, Donepezil treatment was also able to produce a significant upregulation in TREM2 expression in Aβ-treated/AD patients-derived macrophages, together with an increase in its soluble form (sTREM2) in the plasma, dependent on the activation of the α7nAChR. However, even though Donepezil-mediated TREM2 upregulation had an impact on the phagocytic activity of cell lines and human macrophages from healthy controls (it was able to increase the internalization of Aβ), it failed to show any effect on Aβ clearance in human macrophages from AD patients.

Next in order, we observed that Co-ultraPEALut - a nutraceutical showing anti-inflammatory properties in experimental models of AD [11,12] - was able to prevent Aβ-induced peripheral monocytes chemotaxis in a concentration dependent manner. However, studies investigating the mechanism underlying chemotactic inhibition failed to show any involvement of CCR2 in the process.

Concurrently, we were able to demonstrate a substantial increase in TREM2 expression in Aβ-treated macrophages by means of Co-ultraPEALut, probably suggestive of a recovered phagocytic competence. More experiments will be necessary to evaluate if TREM2 increased expression could have a real impact on Aβ internalization.

On the other hand, we explored the possibility that Aβ sequestration in the AD brain, by means of specific immunoglobulins, might reduce its chemotactic power towards peripheral monocytes and increase its phagocytosis by recruited macrophages. In our introductory investigation - focused on the effect of Aβ sequestration by monoclonal antibodies at a concentration comparable to that of NAb detected in the CSF of AD patients [13,14] - we were able to demonstrate that such immunoglobulins were actually able to induce a significant decrease in Aβ-induced migration. However, subsequent analysis invalidated the hypothesis that CCR2 expression could be downregulated by Aβ sequestration and play a role in the modulation of the process.
At the same time, we observed that pathologically low anti-Aβ mAb intrathecal levels are not sufficient to drive phagocytosis of Aβ in peripheral macrophages, nor to enhance their TREM2 expression. Our plan for the future is to verify if the increase of specific anti-Aβ immunoglobulins concentration in the brain of AD patient will deliver the expected protective effect in terms of TREM2 expression and plaque clearance.

The concluding aim of this PhD project was to explore the potential implication of neuroinflammation and the DBI/TSPO system in the agitation/aggression cluster of BPSD. Unfortunately, our results failed to show the expected increase in DBI levels and TSPO monocytic expression in agitated/aggressive AD patients. In addition, peripheral monocytes from A/A patients did not show any difference in terms of chemotactic activity compared to their non-agitated/aggressive counterparts, ruling out the hypothesis of a pathogenic mechanism involving neuroinflammation as the result of an increased monocytic recruitment and neuroinvasion. Of course, these preliminary data need to be confirmed in a larger cohort of patients, possibly encompassing also other symptom profiles in the BPSD spectrum.

Therefore, this work represents a starting point to develop several deepening studies. In the future, we propose to unravel in full the mechanisms underlying Aβ-induced chemotaxis and the modulation of the receptors involved in the process. Moreover, new experiment will be set to complete the characterization of the phagocytic phenotype of peripherally-derived macrophages, for example by direct comparison of

their ability to internalize Aβ with respect to healthy controls. Future investigations will be also aimed at expanding our understanding of the disease-modifying action of the selected compounds, getting leverage on their strengths and strengthening their weakness, in order to validate their therapeutic use. Finally, more experiments will be necessary to understand if neuroinflammation could actually play a role in the pathogenesis of BPSD.

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