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STUDY OF MOLECULAR MECHANISMS AND NEW STRATEGIES AGAINST Aβ CYTOTOXICITY AND NEUROINFLAMMATION IN EX VIVO CELLULAR MODELS FROM ALZHEIMER'S DISEASE PATIENTS

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Abstract

Alzheimer's disease (AD) is a major public health concern and has been identified as a priority for research in Life Science. The two core pathological hallmarks of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles which underlie microglial and neuronal damage, neuroinflammation and cognitive impairment.

Soluble oligomers are the most toxic species of β-amyloid (Aβ) and interact with several protein kinases such as Ras/MAPK and PI3K/AKT pathways, which regulate many cellular processes and cognitive functions. These pathways mediate Aβ toxicity, regulating some molecular mechanisms involved in neuronal degeneration such as cytoskeletal impairment, glutamate excitotoxicity and neuroinflammation. In the last years much attention has been focused on the potential role of natural compounds as neuroprotective agents. Hop (Humulus Lupulus) contains flavonoids, aromatic molecules which have antioxidant, anti-inflammatory and anti-atherogenic properties. In fact, hop extract has anti-aggregating effects on Aβ, and it seems to prevent its production in cultured cells. Aβ induces also the activation of the pattern recognition receptor Nod-like receptor protein 3 (NLRP3) inflammasome complex in microglia and the consequent release of proinflammatory cytokines, playing a pivotal role in AD-associated neuroinflammation. NLRP3 activation results in the release of inflammatory mediators, including ASC protein complexes (ASC specks), IL-1β and IL-18, that facilitate Aβ deposition and neuroinflammation in a self-feeding pathogenic loop. Since specific therapeutical strategies are still lacking, the dampening of the inflammasome assembly and activation could be a new strategy for AD.

The overall focus of this study is to investigate molecular mechanisms involved in neurodegenerative diseases and in neuroinflammation, using peripheral ex vivo cellular models from AD, to check new potential therapeutical targets.

In order to characterize the complex interactions among Aβ, MAPK and AKT signaling, we used fibroblasts from sporadic AD patients with different disease severity. To evaluate any molecular mechanisms that could prevent or modulate Aβ-induced toxicity, the potential cytoprotective effects of Hop extract and related intracellular signaling were also investigated. Fibroblasts provide a useful cellular model for studying AD, since they could be differentiated into patient-specific neural cell lines, using iPSC technologies.

Moreover, particular interest was given to NLRP3-inflammasome activation pathway. We investigated the involvement of NLRP3 inflammasome activation on intracellular pathways and their downstream targets, using a combination of in vitro studies and patient-derived samples. In particular, we used macrophage-derived THP-1 human monocytes and peripheral blood mononuclear cells (PBMC)-derived monocytes from healthy control (HC) subjects and AD patients, to analyse phagocytosis, autophagy and apoptosis modulation and the effects of the nucleoside reverse transcriptase inhibitor Stavudine (D4T), that reduces NLRP3 inflammasome activation blocking the purinergic receptor P2X7R. Furthermore, we analyzed the

NLRP3 inflammasome pathway and the role of the selective NLRP3 inhibitor CRID3, to compare the effects of inflammasome inhibition through two different mechanisms. At this purpose, HC and AD-derived monocytes were differentiated into microglia-like cells (MDMIs) and characterized for myeloid surface and intracellular proteins expression. Key microglia functions such as inflammatory cytokines release, Aβ phagocytosis and degradation were evaluated upon exposure to NLRP3 inflammasome activators with or without CRID3.

MDMIs reflected many features of microglia and, as fibroblasts-derived iPSCs, they are attractive cellular models helpful to understand AD pathogenesis, identify therapeutic targets and allow large-scale drug screening of the novel therapeutic candidates.

Chapter 1

Alzheimer's Disease

1. Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia in the elderly accounting for 50–60% of all cases[1] with a prevalence of 5% after 65 years of age, increasing to about 30 % in people aged 85 years or older [2]. AD is a progressive brain disorder affecting regions of the brain that control memory and cognitive functions, gradually destroying a person's memory and ability to learn, to reason, to communicate, and to carry out daily activities [3]. The classic clinical features of AD are an amnesic type of memory impairment, deterioration of language and visuospatial deficits [4]. It is often accompanied by personality changes and behavioral disturbances [5]. Many molecular lesions have been detected in AD, but the main hallmark is an accumulation of misfolded proteins in the aging brain results in oxidative and inflammatory damage, which in turn leads to energy failure and synaptic dysfunction [6]. AD brains show two characteristic lesions: extracellular deposits of β-amyloid (Aβ) peptides, called neuritic or senile plaques, and intracellular neurofibrillary tangles (NFTs) of hyperphosporylated tau protein [7]. The downstream consequences of these pathological processes include neurodegeneration with synaptic and neuronal loss leading to macroscopic atrophy [8]. This loss is spread in neocortex, in the hippocampus and in the entorhinal cortex including, the cholinergic nucleus of Meynert [9].

Despite Aβ and tau accumulation being always present in the brain of AD patients, they are not considered absolute markers for the pathology. However, there are many additional cellular pathways, processes, and molecules involved in AD pathogenesis that play important roles in the disease [10]. Since AD progression tends to be slow and gradually worsens over several years, clinical assessment and cognitive testing, remains critical for the diagnosis [11].

1.2 Pathological subtypes

Two different pathological subtypes of AD based on the age of onset have been identified: early-onset AD (EOAD) and late-onset AD (LOAD). Familial or early onset dementia accounts for approximately 1% to 6% of all cases, includes patients with onset at 40-50 years of age [12,13] and it is associated to autosomal dominant missense mutations in different genes [14,15]. The sporadic or LOAD form, include the 95% of all AD cases with an age at onset later than 65 years and develops as a result of multiple factors, such as environment and genetic predisposition [16].

- Early-onset dementia

Missense mutations that are causative of EOAD have been identified in three genes that are essential for the generation of Aβ peptides: the APP gene, located on chromosome 21 and two homologous genes, located on chromosome 14 and 1 respectively, that encode the catalytic subunit of γ-secretase, presenilin-

1 (PSEN1) and presenilin-2 (PSEN2) [17,18]. Most of the mutations, affect the biophysical properties of the Aβ peptide and they consistently increase the toxic amyloid potential of the protein, thereby leading an impairment in the amyloidogenic pathway and increasing the tendency of Aβ to oligomerize [19].

- Late-onset Alzheimer's disease (LOAD)

The etiology of LOAD is complex, probably involving several disease susceptibility and modifying genes, together with environmental factors [20]. The ApoE gene located on chromosome 19q13 has been identified as a strong LOAD risk factor. This gene encode for apolipoprotein E (ApoE), a 299-amino-acids-long glycoprotein normally produced by astrocytes and microglia but that could also be expressed by neurons, following stress or cellular damage [21]. ApoE is a component of lipoprotein particles and it is associated with cholesterol, triglycerides and phospholipids. Three variants of ApoE (ApoEε2, ApoEε3 and ApoEε4), have been found to modify the risk of LOAD [21]. These variants confer conformational differences, which influence the shape of ApoE protein and participate in the risk of developing AD [22]. ApoE ε4 is most highly associated with AD for individuals with a family history of dementia, and this association is highest for homozygous individuals [23]. ApoE ε4 is associated to decreased Aβ clearance and increased amyloid fibril formation [24]. Moreover, the ApoE4 fragments accumulate in neurofibrillary tangles and amyloid plaques, disrupt cytoskeletal structure, and impair mitochondrial function [25].

Large-scale genome-wide association studies (GWASs) have identified single-nucleotide polymorphisms in multiple genes which are associated with AD: ABCA7, B1N1, TREM2, CD33, CLU, CRI, EPHA1, MS4A, PICALM and MEF2C genes. The proteins coded by the candidate genes participate in a variety of cellular processes such as oxidative balance, protein metabolism, cholesterol metabolism and synaptic function [26,27]. Recently, microglia associated genes, including TREM2, TYROBP and CR1, have been identified as risk factors for developing AD [28]. In particular, the new risk variant in Triggering receptor expressed on myeloid cells 2 (TREM2) R62H [28] R47H and H157Y, were significantly associated with AD risk and provide evidence that the microglia mediated innate immune response and alteration of TREM2 signaling pathways, contribute directly to AD development [29].

Beyond genetics, the major risk factor for LOAD is age, with a sharp increase in incidence after 60 years [30]. However, LOAD has also strong environmental and metabolic components that could facilitate its onset. The main metabolic and nongenetic risk factors include hypercholesterolemia obesity, hypertension, type 2 diabetes mellitus [31] and hyperhomocysteinemia [32].

There is a link between obesity and AD which alters brain homeostasis causing inflammation and oxidative stress [33]. Obese individuals have high circulating levels of lipids and glucose and chronic inflammation, which increase Aβ aggregation [34].

Diabetes has been linked to an increased risk of AD [35]. Several mechanisms for this association are suggested, including insulin resistance and insulin deficiency, impaired insulin receptors, toxicity of hyperglycemia, adverse effects due to advanced glycation end products, cerebrovascular damage, and inflammation [36]. Lastly, hyperhomocysteinemia and hypertension seem to be predisposing factors to AD pathology, which has been linked to dysfunction in the blood-brain barrier (BBB), brain atrophy as well as NFTs formation [37,38].

1.3 Aetiopathogenesis

AD etiopathogenesis is not yet completely understood, the responsible event triggering the neurodegenerative process is still unknown. In order to explain its pathogenesis, several and different hypotheses have been formulated. The phenomena most studied to clarify the pathogenetic mechanisms are: Aβ peptide, Tau protein hyperphosphorylation, oxidative stress, and neuroinflammation [6].

- The Amyloid Cascade Hypothesis

This hypothesis proposes that progressive cerebral accumulation of Aβ initiates a complex multicellular cascade that includes microgliosis, neuritis dystrophy, neuronal dysfunction and loss [7].

The accumulation of Aβ peptides originates from a series of anomalies in both the productive and secretive pathway of amyloid precursor protein (APP) [39]. In the brain, the main form of APP is a 695 amino acid type I membrane protein that, in physiological conditions, undergoes a non-amyloidogenic processing by α-secretases [40] such as ADAM 9, ADAM10 and ADAM17 [41], and by γ-secretase[40]. The γ-secretase complex consists of four essential subunits: presenilin-1 (PSEN-1) or presenilin-2 (PSEN-2), nicastrin, anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN- 2) [42]. The first cleavage results in the secretion of the large and soluble extracellular domain of APP (sAPPα) [41] which has been shown to be neuroprotective, and in a C83 C-terminal fragment [43]. Then, it is cleaved by the enzyme γ-secretase, which generates two non-toxic fragments: p3-peptide and a small APP intracellular domain (AICD) portion [44].

However, APP may undergo a second amyloidogenic processing. Aβ production starts with the cleavage of APP by the β-secretase BACE1 (β-site APP-cleaving enzyme 1), which cut the APP generating a soluble APPβ peptide (sAPPβ) and a membrane-bound carboxyl terminal fragment (CTF), C99 [45]. Subsequently, C99 is cleaved by γ-secretase to generate AICD and β-amyloid [46]. The γ-secretase processing is not precise,

and lead to the liberation of different Aβ peptides; while the majority end at amino acid 40 ($Aβ_{40}$), a small proportion end at amino acid 42 ($Aβ_{42}$) [47].

Although, both these two peptides are the main components of the senile plaques in the parenchyma of patients brain, $Aβ_{42}$ is more prone to aggregate but also far more neurotoxic than $Aβ_{40}$ [48]. In AD, $Aβ_{42}$ monomers aggregate into progressively larger polymers, known as oligomers which represent the most toxic species of Aβ [49].

Soluble oligomers interfere with normal synaptic functions and contribute to the early memory loss and cognitive decline [50]. It is known that oligomers trigger mitochondrial dysfunctions and oxidative stress [51], inflammation [52], impaired cholinergic transmission [53], disruption of synaptic plasticity, increased plasma membranes permeability and alteration of excitatory–inhibitory balance[54], that lead to NMDA receptor overstimulation and excitotoxicity [55]. Moreover, they can lead to hydroxyl radicals generation that cause lipid peroxidation, impairing of ATPases and glucose transporters [56] and calcium homeostasis deregulation [57]. However, these detrimental effects seem to be related to Aβ oligomers various sizes [58] and their critical concentration [59], which acts as a threshold to originate the downstream signaling, together with hippocampal atrophy and dementia [60]. Moreover, AD patients show an impairment and a decreasing of Aβ clearance, which is one of the main causes of the accumulation of the peptide [61]. The proposed mechanism assumes that cerebral Aβ concentrations increase because of ageing and due a reduced expression of lipoprotein receptor-related protein-1 (LRP-1) [62], that results in decreased Aβ degradation [63]. Enhancing LRP-1 expression and functions in neurons and astrocytes could be an effective strategy to counteract amyloid deposition [64]. Nevertheless, the aggregation of this peptide is not the only pathogenic trigger, the inefficient removal of Aβ peptides can generate toxic species that contribute to AD progression.

- Tau hyperphosphorylation

The second pathological hallmark of AD is characterized by the presence neurofibrillary tangles caused by hyperphosphorylation and aggregation of tau protein. Tau is a neuronal microtubule-associated protein, which is normally present in neuronal axons and it is responsible for stabilization of neuronal microtubules, neurite outgrowth and transport of vesicles, organelles, and protein complexes [65,66]. Six tau isoforms are expressed in the adult human brain, generated by the alternative mRNA splicing of microtubule-associated protein tau (MAPT) gene [67]. Isoforms can also be categorized depending on whether they contain three or four C-terminal repeat domains, 3R or 4R, respectively. In the healthy brain, these isoforms are found in equal amounts [68]. Alterations in the 4R:3R tau isoform ratio, often caused by mutations in the MAPT gene, trigger pathological tau aggregation and NFT formation [69]. As a result of

dysfunctional phosphorylation process, tau is abnormally hyperphosphorylated in AD brains. Hyperphosphorylated tau is unable to interact with microtubules, which disrupts axonal transport [70] and can drive the misplacement of tau into postsynaptic spines, resulting in synaptic dysfunction and neurodegeneration [71].

As has been observed for Aβ, tau deposition correlates with disease progression [72] and recruits soluble monomeric species to the already formed NFTs. Tau aggregates can be secreted from neurons and aggregation can be transmitted from neuron to neuron [73].

Some evidences suggest that tau oligomerization is not only a consequence of Aβ pathology, but also a critical mediator of the toxic effects [74]. However, there is a link between Aβ toxicity and tau pathology, but the underlying mechanisms have not been elucidated yet [75].

Some studies suggested that tau might mediate Aβ toxicity [68,75,76]. As has been shown, the reduction in tau levels can alleviate memory loss and prevents behavioral deficit in the AD mouse model [77]. Recent studies showed that tau deficiency, attenuate Aβ toxicity and protect neurons from Aβ-induced cell death indicating that tau is a mediator of Aβ toxicity [78].

- Cholinergic hypothesis

The cholinergic hypothesis focuses the attention on the role of acetylcholine in the onset of the disease. Essentially, it states that the reduction of acetylcholine (ACh) in presynaptic cholinergic terminals of the hippocampus and the neocortex regions, caused by loss of cholinergic function in the basal forebrain nuclei, influences all aspects of cognition and behavior and contributes significantly to the cognitive decline associated with aging and AD [79,80].

Deficits in the cholinergic transmission are associated to impaired choline uptake and acetylcholine release, alteration of nicotinic and muscarinic receptors expression [81,82] and these deficits are linked to the down-regulation of specific enzymes such as acetyltransferase and acetylcholinesterase, which contribute to the cognitive impairment onset [83].

Cholinergic abnormalities might affect the glutamatergic system, which is also impaired in AD. Excessive activation of NMDA receptor due to Aβ is implicated in the degenerative process of cholinergic neurons [84]. In light of the interplay between the glutamatergic and cholinergic pathways, the development of pharmacological strategies targeting both the cholinergic and the glutamatergic system, has been examined in several preclinical studies [85,86].

- Other pathogenetic factors

AD is a complex and multifactorial disease. Beyond deposition of amyloid plaques, tau hyperphosphorylation and cholinergic dysfunction, some other processes that could be causative factors have been proposed. One of those is oxidative stress, which is involved in several neurodegenerative disorders [87,88]. Oxidative stress occurs early in AD, significantly before the development of the pathology hallmarks and one of the causes seems to be the deposition of Aβ insoluble forms [89]. Oxidative damage implies the development of reactive oxygen species (ROS), which can modify and disrupt the structure of proteins, lead to lipids peroxidation and alter nucleic acids, increasing apoptosis [90,91]. Moreover, oxidative stress affects neuronal mitochondria, disrupting their functions and leading cells to death [92].

Oxidative stress can be also due to the glutamatergic system impairment. Glutamate is the major excitatory neurotransmitter in the central nervous system and is involved in synaptic transmission, neuronal growth and differentiation and also, in learning and memory [93].

Aβ accumulation resulting in disruption of glutamatergic synaptic transmission, altering AMPA and NMDA receptors [94]. Moreover, neurodegeneration in AD is associated both with aberrant glutamate transporter expression and activity (EAAT1 and EAAT2) and with the reduction in glutamine synthesis [95]. Alterations in glutamate transport can lead to an increase of extraneuronal glutamate, resulting in stimulation of NMDA receptors and excitotoxicity processes due to abnormal Ca^{2+} release, which is associated with loss of dendritic spines and neuronal degeneration [84,96].

Beyond these cellular mechanisms, another important trigger of the disease is neuroinflammation, that impairs function connectivity leading to cognitive decline and it strongly depends on activated microglia [9798]. Hence, AD is a complex and multifactorial disease that is not attributable to a single specific trigger factor. Therefore, to unravel this intricate scenario is mandatory, in order to develop effective therapies to stop or reverse AD progression.

1.4 Diagnosis

The definitive diagnosis of AD is possible post-mortem only, after a histopathological examination of its main pathological hallmarks with a brain biopsy [99]. Currently, the most widely used diagnostic criteria are the Diagnostic and Statistics Manual of Mental Disorders (DSM-V) criteria for the differential diagnosis of dementia, together with specific guidelines established by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ARDRA) [100]. These criteria allow to designate the clinical diagnosis of AD only when patients display significant functional disability and a strong cognitive impairment, which interfere with normal and daily activities, and behavioral disturbances not due to other neuropsychiatric conditions [101].

The pathology displays different degrees of severity, identified as a preclinical phase, an amnestic Mild

Cognitive Impairment (aMCI) stage and, AD dementia [102]. The preclinical stage starts early in the lifetime of an individual and is totally asymptomatic at the beginning. Slowly, cognitive decline starts to interfere with everyday activities, compromising functional autonomy until the end of the preclinical stage, when the impairment can be observed [103,104]. The Mild cognitive impairment (MCI), is defined by performance that is lower than normal on objective neuropsychological cognition tests, although the subject still maintains daily functions and activities [104]. MCI can be categorized into "amnestic" MCI, characterized by reduced memory performance and "non amnestic" MCI, in which there are reduced other cognitive performances. However, MCI does not always convert to dementia and a patient's cognitive status may revert to healthy condition [105,106].

Useful for AD diagnosis, is the evaluation of anamnesis for cognitive decline and impairment in daily activities, examination of medical history, laboratory testing, and brain imaging [107]. Several screening tools are available for detecting dementia such as Montreal Cognitive Assessment (MoCA) [108], Alzheimer's Disease Assessment Scale, Cognitive subscale (ADAS-Cog), Disability Assessment of Dementia (DAD) and Mini-Mental State Examination (MMSE) [109–111]. This one, is the most widely used tool to assess cognitive function, which is reported as an MMSE total score, which correlates with disease progression [112]. It consists of 16 items with a score ranging from 30 points for healthy individuals, to 0 points for severe cognitive impairment. So, according to the obtained score, it is possible to identify mild (MMSE score 21–26); moderate (MMSE score 15-20), moderately severe and severe dementia (MMSE score <15) to cover the full spectrum of AD severity [113,114].

- Imaging and biomarkers

However, these standards are not solid enough to formulate a diagnosis of probable AD and often requires additional tools, such as evaluation of cerebrospinal fluid (CSF) biomarkers and neuroimaging techniques, which include magnetic resonance imaging (MRI) and Positron Emission Tomography (PET) [101,115]. MRI provides high-resolution cerebral pictures, allowing the characterization of regional brain abnormalities in anatomical and functional connectivity networks. AD patients show atrophy of the medium temporal lobe, ventricular enlargement and lower total brain volume [116,117]. While, PET allows the in vivo visualization of changes in brain metabolism may precede structural brain alterations [102]. Using 18-fluoro-deoxyglucose (18F-FDG) as a tracer, this technique can reveal the lower glucose consumption of AD brains, compared to healthy subjects [118,119]. FDG-PET also allows a differential diagnosis with other types of dementia, in particular frontotemporal dementia, due to the different localization of glucose uptake alterations [120]. Moreover, using Pittsburgh-Compound (PIB) as a marker, PET allows to visualize in vivo the brain areas where Aβ fibrillar accumulates. AD patients shown a higher tracer uptake than controls and the localization is comparable to the pattern of amyloid plaque deposition observed in post-mortem

examinations [121,122]. In addition, PET might be used to track tau protein and neurofibrillary tangles [123]. Even though they are useful, brain imaging techniques are very expensive and require skilled technicians, so these examinations are not the most suitable screening methods for large groups of patients. Hence, clinical examinations are often accompanied by biomarker analysis, which can be very helpful for early identification of the disease, given that the pathological process starts decades before the onset of dementia [101,124]

The core biomarkers that have been developed, reflect amyloid, neurofibrillary tangle pathology, and axonal degeneration [125]. AD biomarkers are divided into 3 binary classes and classified with A/T/N classification system, where "A" refers to the value of $A\beta_{42}$ levels, "T" refers to tau, and "N" to neurodegeneration or neuronal injury. Abnormal values in all 3 of these are strongly associated with worse cognitive symptoms [126,127].

The assessment in CSF of $A\beta_{1-42}$ levels, which reflects fibrillar amyloid, total tau (related to neuronal degeneration) and phosphorylated tau in position 181 (p-tau_{181}, which represent a marker of tangle pathology) levels are considered the best direct indicators of AD pathology available to date with an accuracy around 90% [125,128,129]. Compared to healthy controls, AD patients exhibit decreased $A\beta_{1-42}$ levels, which is related to amyloid plaque load within the brain [130,131], while p-tau_{181} and t-tau are present in high levels, and it is related to neocortical neurofibrillary tangle pathology, as well as with the rate of hippocampal atrophy in the brain [129,132]. Moreover, high CSF t-tau has also been associated with fast progression from MCI to AD [133]. Furthermore, p-tau/$A\beta_{1-42}$ and t-tau/$A\beta_{1-42}$ ratios have been shown to be predictive of cognitive decline in MCI and mild demented subjects [134,135].

During the last years, other CSF biomarkers have been searched in order to increase diagnostic accuracy. They are linked to AD pathogenetic processes, such as amyloid production and deposition, neurofibrillary tangles formation and neuronal degeneration. Noteworthy are BACE1 [136], sAPPα [137], Aβ40 and other Aβ truncated forms[138], p-tau_{231} [139], F2-isoprostane which is related to neurons free radicals mediated injury [140], microglia derived cytokines and chemokines and inflammatory markers [141,142].

Recently, a soluble form of TREM2 (sTREM2) has been identified as a potential AD biomarker, since high levels of sTREM2 has been detected in the preclinical stages of AD and positively correlates with the amounts of total and phosphorylated tau in the CSF [143–145]. Nevertheless, further evidence is needed to support the role of sTREM2 as marker of neuroinflammatory response in AD.

In addition, despite the fact that these substances are easily measurable with commercially available kits, these techniques are subject to significant differences in the measurement of biomarkers in different research laboratories. Moreover, there is still a lack of approved standards for quantitative analysis of imaging tests for AD [115,125]. Even though these molecules are highly detectable in CSF, obtaining this biological fluid presents some drawbacks. In fact, the lumbar puncture for its collection is very invasive

and is not suitable for either initial diagnosis or follow-up checks. For this reason, several studies have focused on peripheral blood, which is inexpensive and easily accessible. Many studies have examined Aβ plasma levels as a biomarker for AD, obtaining conflicting results, may due to the fact that Aβ detected in plasma, is derived from peripheral tissues and does not reflect the turnover or metabolism of Aβ in the brain [129,146]. Recently, plasma neurofilament light chain (NFL) have been proposed as a blood-based biomarker for neurodegeneration in AD. Compared to healthy subjects, high NFL concentration in AD patients even in prodromal disease have been reported, and it correlates with the disease hallmarks [147,148]. However, increased NFL plasma levels are also found in several other neurodegenerative disorders, meaning that it lacks disease specificity for AD [149]. Future longitudinal studies are needed to validate whether NFL plasma levels may be represents useful tool for AD diagnosis.

1.5 Pharmacological therapy

Nowadays, the therapeutic approach is aimed at delaying the progression of the disease, improving cognitive function and reducing depressive symptoms and behavioral dysfunctions. Indeed, there are no definitive pharmacological treatments available to reverse or stop the progression of AD.

- Acetylcholinesterase inhibitors

First class of Food and Drugs Administration (FDA) approved drugs for AD treatment are acetylcholinesterase (AChEI) inhibitors, such as donepezil, rivastigmine and galantamine, which promote higher levels of ACh in the synaptic cleft by inhibiting its degrading enzyme acetylcholinesterase [86]. Tacrine had a short half-life and it is associated to hepatotoxicity, given that it is rarely used and no longer available in many countries [150]. AChEI exert their pharmacological action by restoring cholinergic transmission, which is impaired due to Aβ accumulation [151]. These drugs partially ameliorate cognitive symptoms and enhance quality of life. Moreover, these compounds are well tolerated although they have dose-dependent side effects [152].

- Anticholinergic drugs

Although cholinesterase inhibitors are not effective in slowing AD progression, other drugs targeting the muscarinic and nicotinic receptors might still produce promising results. These drugs seem to ameliorate cognitive decline in prodromal AD subjects, even though they display several side effects [153,154].
A chronic exposure to anticholinergic medications is not recommended, since they have been shown to increase AD risk and this outcome is more pronounced in older adults, due to decreased acetylcholine synthesis [154,155].

- NMDA antagonist - Memantine

Memantine is a low to moderate affinity noncompetitive NMDA receptor antagonist, which was approved by FDA for the treatment of moderate-to-severe AD [156]. This drug can protect neurons from glutamate-mediated toxicity due to Aβ by attenuating phosphorylation of tau through a decrease in glycogen synthase kinase-3beta (GSK-3β) activity via the PI-3K/AKT kinase-dependent pathway [157]. The drug is well tolerated and there is a low incidence of side effects. Moreover, it can be administered alone or in combination with AChEI [158].

- Serotonin receptors antagonists

Lastly, several serotonin receptor antagonists are in clinical development as adjunct therapy to AChEI [159]. Much attention has been focused on serotonin receptors 6 (5-HT 6), which are localized in brain regions associated with cognition and behavior and their inhibition, seem to improve cholinergic transmission [160].

- Aetiology-based strategies

Development of disease-modifying therapeutics is urgently needed for treating AD. Much attention has been focused on therapeutic strategies which aim to interfere with amyloid deposition, either influencing its formation [161]. A number of small molecules were designed to modify the amyloidogenic pathway, inhibiting the enzyme β-secretase and γ-secretases, that are required for Aβ production [162], whereas other molecules were developed to induce the up-regulation of α-secretases like ADAM10 [163].
Unfortunately, clinical trials with BACE1 inhibitors were unsuccessful because of their ineffectiveness in human trials or their non-specificity for receptors [164]. In addition, issues in drug delivery and toxicity have been occurred. Interfering with γ-secretase activity has shown severe side effects, such as alterations in axonal trafficking and signaling [165,166]. Conversely, the up-regulation of α-secretase action has been revealed potential neuroprotective, reducing formation of Aβ and increasing the assembly of αAPPs [167].
Furthermore, anti-aggregation pharmacological treatments have been developed, to avoid Aβ oligomerization stabilizing Aβ monomers. Unfortunately, these compounds failed in clinical trials, displaying low CNS bioavailability and important side effects [168].
Other pharmacological strategies have targeted tau, trying to inhibit its hyper-phosphorylation and aggregation, without showing any clinical benefit [161]. In recent years, different therapeutic strategies in anti-tau immunotherapy have been developed and have encouraged new clinical trials but, further studies are needed [169]. Lastly, statins appeared beneficial to dementia because of their role in reducing cholesterol, but they failed to prevent cognitive decline [170,171].

- Immunotherapy

In recent years, immunization strategies have been developed to remove monomeric and aggregated Aβ and to prevent generation of these aggregates. This anti-Aβ approach includes both active vaccines to stimulate the immune system to produce its own antibodies, and passive immunization through the administration of exogenous antibodies [172].

Initial experience with active vaccines demonstrated that, $A\beta_{42}$ when combined with different adjuvants, reduced both plaques and cognitive deficits in vivo [173,174], given that clinical trials with the different vaccines have been started, but most of them were discontinued following the occurrence of a harmful immune responses and T-cells mediated meningoencephalitis in some cases [175,176].

On the other hand, passive immunization strategies have been explored. These are based on peripheral infusion of monoclonal or polyclonal antibodies, targeting Aβ to induce its clearance and hence, prevents oligomer formation [171], which results in an improvement of cognitive functions and ameliorated behavior [177,178]. Unfortunately, antibody-based immunotherapy against Aβ has so far been unsuccessful. A peripheral "sink effect" has been theorized and it is hypothesized that, the antibodies in the plasma extract Aβ from the brain by altering the Aβ equilibrium across the BBB through the lipoprotein receptors [179]. In addiction amyloid-related imaging abnormalities (ARIA) have been reported, which include MRI anomalies due to the presence of cerebral edema and micro hemorrhages of the vessels [180]. The lack of success may have been due to the inability of the antibodies to adequately engage the proper target in the brain, besides the expensive costs, repeated injections needed and several side effects on immune system activation [179,181,182] occurred.

Recently, several studies suggested that, targeting TREM2 could decrease AD-related pathologies [183]. At this purpose, monoclonal antibodies raised against TREM2 were screened and tested, in order to boost TREM2 expression and signaling [184,185]. Have been reported that TREM2 antibodies enhanced microglial survival and uptake of Aβ peptides in cultured cells, while they helped microglia clearing Aβ plaques in amyloidosis mouse model [185]. Nevertheless, further studies are needed to better understand whether enhancing TREM2 activity, could be a novel and efficacious therapeutic approach for AD.

Since, the pathogenesis of AD entails multiple factors such as Aβ, tau, neuroinflammation, oxidative stress and ApoE [2], multi-targeted drugs and combination therapies may have a greater chance of future clinical therapeutic success [186,187].

1.6 Non-Pharmacological Strategies

Several studies have focused on preventive drug-free strategies, which can be associated with both lifestyles and diet.

- Lifestyle

Psychosocial factors and an actively lifestyle can reduce the risk of AD and dementia. High level of education, mentally demanding activities, physical exercise and socialization have a protective role on aging-related decline and may enhance overall cognitive functions [188,189].

Physical exercise in particular, is thought to have positive effects on brain health, promoting brain plasticity, and inducing neurotrophic factors release that are relevant for the maintenance of cognitive functions [190,191]. Moreover, regular physical activity was reported to be associated with a delay in onset of dementia and AD [192,193].

- Diet and natural compounds

Currently much attention has been focused on the potential role of natural compunds as neuroprotective agents, to attenuate or suppress inflammation, oxidative stress and Aβ-mediated cytotoxicity [194]. Among them, antioxidant vitamins (C and E), folate, ω-3 fatty acids, vitamins B and polyphenols, which have been suggested to aid in the prevention of degenerative diseases, including AD [195–197]. Also, resveratrol is an anti-aging polyphenol derived from grapes. As have been reported, it ameliorate mitochondrial dysfunction due to its antioxidant effects, [198] promotes intracellular degradation of Aβ [199], modulates neuroinflammation, and induces adaptive immunity [200]. Curcumin is another natural polyphenolic compound which have beneficial effects on neuronal protection. Has been shown, it reduces amyloid burden by immunomodulating microglia [201], decrease pro-inflammatory cytokines release attenuating NLRP3 inflammasome activation [202,203].

Hop (Humulus Lupulus) contains flavonoids, aromatic molecules which have antioxidant, antinflammatory and antiatherogenic properties [204]. Hop flavonoids inhibit Aβ production, and its oral administration, suppress neuroinflammation and ameliorates Aβ deposition, leading to an improvement of memory and cognitive functions in AD mice models [205,206], may due to its ability to recognize and bind Aβ oligomers, preventing their aggregation and their neurotoxicity [207]. Moreover, have been shown that hop modulates specific intracellular signaling pathways, such as ERK1/2-CREB pathway and the PI3-kinase-mTOR cascade, underlying neuronal survival and cognitive performances [197,208]. Also, coffee has antioxidant properties and may has a protective role against cognitive deterioration, decreasing senile plaques and Aβ levels in AD mouse models [209]. Epidemiologic studies indicate that its consumption is associated with lower AD risk

[210,211], may due to the presence of several bioactive compounds such as chlorogenic acid (CGA), quercetin [212] and melanoidins. CGA is a phenolic compound predominantly present in green coffee beans while melanoidins occur during the coffee roasting process [213]. These compounds have been reported, are able to hinder Aβ peptide fibrillation, prevent cell death due to oxidative stress and modulate autophagic pathways [214]. Is thought that coffee may exert its neuroprotective effects toward the modulation of antioxidative signaling pathways, stimulation of pro-survival cascades and inhibition of pro-apoptotic pathways [215,216].

Therefore, a definitive therapy for AD has not been discovered yet and both pharmacological and non-pharmacological therapies, offer some symptomatic relief or are aimed to preventing them. Since pathogenetic processes start much earlier the dementia onset, it is mandatory to improve techniques for an early diagnosis. In fact, reversing cognitive decline is more problematic as AD progresses, because the mental decline is too extended. Hence, treatments should be administered in pre-clinical phases, in order to increase their efficacy and minimize brain impairment [217]. Moreover, the identification of changes in biomarkers levels during the pathology and in asymptomatic patients at risk of AD, it would allow a better diagnosis as well as a prognosis hypothesis [218].
For these reasons, it is mandatory to continue to explore not only the etiology of AD, but also a causal treatment which may slow disease progression or prevent clinical symptoms onset.

Chapter 2

Intracellular signal transduction

2. Intracellular signaling pathways

The intracellular signal transduction mediates a wide range of cellular processes and biological functions. Alterations in intracellular signaling pathways have been found to play key roles in the pathogenesis of various neurodegenerative disorders such as AD, also in relation to the Aβ impact on protein kinases [219].

2.1 MAPK - mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases which coordinately regulate various cellular activities including gene expression, metabolism, proliferation, survival and apoptosis. MAPK pathways can be activated by a wide variety of stimuli acting through diverse receptor families [220]. MAPK signaling cascades are composed of at least three hierarchically sequential kinase components: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK. MAP3Ks phosphorylate and activate MAP2Ks, which in turn phosphorylate and activate MAPKs [221]. To date, seven distinct groups of MAPKs have been characterized and the most extensively studied are: extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) and p38 MAPK, each of which exists in several isoforms [219,222]. MAPKs interact with both upstream activators, regulatory proteins and substrates through conserved clusters of acidic amino acids T-X-Y, within the activation loop, in which MAPKs are activated by concomitant Thr and Tyr phosphorylation [222]. However, this sequence is replaced by a single phospho-acceptor residue within an S-E-G motif in atypical MAPKs such as ERK3, ERK4, ERK7 and Nemo-like kinase (NLK) [223,224]. Nevertheless, the regulatory mechanisms and physiological functions of atypical MAPK is poorly understood [225].

- Extracellular signal-regulated kinase 1/2 - ERK 1/2

ERK1/2 cascade is a member of MAPK that transmits signals from extracellular stimuli such as growth factors, hormones and neurotransmitters by which regulates proliferation, differentiation, cell survival, learning, apoptosis, and oncogenic transformation [226]. ERK is predominantly found in two isoforms, ERK1 (p44) and ERK2 (p42), respectively and are expressed in all tissues [227]. There are also several atypical forms of ERK, including ERK3, ERK4, ERK5 and ERK7 [228]. ERK1/2 proteins are serine/threonine protein kinases that are a component of the RAS/RAF/MEK/ERK signaling cascade [229], which activation starts from membrane tyrosine receptors and involve the recruiting of adaptive proteins, such as Grb2 and the exchange factors SOS (son of sevenless), which in turn induce Ras activation [230,231]. Thus, binds GTP and transmits the signal through the activation of Raf protein kinases, which in turn activate the MAPK responsible for Thr183 and Tyr185 phosphorylation at ERK1/2 regulatory sites [230,232].

Although numerous studies have associated the ERK cascade to a wide range of cellular processes including proliferation, survival, autophagy and apoptosis [233,234], several lines of evidence support a role for the ERK signaling pathway in the pathogenesis of AD. Aβ can lead to overactivation of the RAS/ERK signaling cascade, which implies a pathologic link between Aβ and altered ERK phosphorylation [235]. High levels of phosphorylated ERK (p-ERK) have been found in degenerating neurons, in APP mouse models [235], in CNS from patients at early stages of AD [236] and in tangled neurons, suggesting also its involvement in tau hyperphosphorylation [237,238]. Activation of ERKs, which is more often associated with neuroprotective processes, can participate in neuronal apoptosis that is induced by oxidative stress and glutamate induced Ca^{2+} release in neurons [239]. However, ERK activation can lead to neurodegeneration or neuroprotection depending on the cellular conditions and interplay with other signaling pathways [240].
Indeed, ERK promotes the non-amyloidogenic APP processing by α-secretase and negatively modulates BACE1 expression and activity, leading to reduced Aβ generation [240,241]. Moreover, ERK1/2 signaling pathway is involved in synaptic plasticity and memory formation [242] through cAMP response element binding protein (CREB) phosphorylation, which is linked to long-term memory acquisition and consolidation [243]. ERK1/2 levels are detectable in human CSF and are correlated with both high t-tau and p-tau levels and decreased $Aβ_{1-42}$ concentration. Therefore, ERK1/2 liquor dosage turned out to be not enough solid as a predictive diagnostic tool [244].

- c-Jun N-terminal Kinases - JNK (SAPK)

JNK is one of the identified family of stress activated MAP kinases (SAPK), are encoded by three genes which are spliced in more than 10 forms: jnk1 (MAPK8), jnk2 (MAPK9) which have a broad tissue distribution, while jnk3 (MAPK10) is mainly expressed in neurons [245]. JNK play a central role in stress signaling pathways implicated in gene expression, neuronal plasticity, regeneration, cell death, and regulation of cellular senescence. Its activation occurs in response to different stressing factors, including cytokines release, oxidative stress, unfolded protein response signals and Aβ peptides [229,246].
Activation of the JNK pathway relies on the coordinated interplay with JNK interacting proteins (JIP), which act as of scaffold proteins and lead to the phosphorylation of a Tyr-Pro-Thr motif in the activation loops [247]. The main cellular substrate activated by JNK phosphorylation is c-Jun, which in turn interact with JunB, JunD, c-Fos, and ATF constituting the activator protein-1 transcription factor (AP-1) thus, regulating the cellular stress-response [248]. Moreover, JNK phosphorylate pro-apoptotic proteins such as BIM and BMF [249], resulting in activation of caspases, and both anti-apoptotic proteins, such as Bcl-2 family, reducing their activity [250]. However, the role of JNK in apoptosis is controversial and depends on cell type and the nature of the death stimulus [251].
Activation of JNK is implicated in multiple pathological features of neurodegenerative diseases including AD. JNK has been reported to participate in Aβ generation through elevation of BACE1, inducing

amyloidogenic APP cleavage [240]. Moreover, Aβ mediated oxidative stress lead to JNK cascade activation, resulting in increased levels of senile plaques and NFT [252] and neuronal death [253]. Furthermore, high levels of phosphorylated JNK (p-JNK) have been reported, in human post-mortem brain samples from AD patients, which colocalized with Aβ plaque and NFT [254]. In particular, have been shown that JNK3 is highly expressed and activated in brain and CSF from AD patients, which is correlated with the rate of cognitive decline [255]. Given that, JNK potentially contributes to neuroinflammation and synaptic dysfunction associated with AD, suggesting that JNK activation represents a potential therapeutic target. At this purpose, JNK inhibitors have been developed. In particular, SP600125 selective inhibitor toward JNK1, JNK2, and JNK3 isoform, has been shown to be effective in slowing down disease progression, reducing, multiple AD pathological features, and ameliorating cognitive deficits in AD animal models [256,257]. However, direct inhibition of JNK isoforms in specific tissues is still an open challenge.

- p-38 MAPK (SAPK)

P38 was initially identified as a 38 kDa protein that was phosphorylated on Tyr in response to environmental stress. There are four p38 isoforms (α, β, γ and δ), which are activated following oxidative stress and pro-inflammatory cytokines [227]. In particular, TNFα and IL-1β activate p38 isoforms by promoting the recruitment of TRAF adaptor proteins, that promote activation of MKK3 and MKK6 which phosphorylate Thr180 and Tyr182 residues on the conserved Thr-Gly-Tyr motif in p38 activation loop [258]. In addiction, p38 can be also phosphorylated by MKK4, an activator shared with JNK pathway [222]. Moreover, an additional alternative p38 activation pathway involve TAB1 (TAK1-binding protein 1), which can bind only to p38α isoform, and induces its autophosphorylation in the activation loop [259]. Thus, might contribute to p38α regulation in myeloid cells [260]. Activated p38, phosphorylates a great variety of substrates, mostly kinases and transcription factors as CREB, ATF1, NF-κB and STAT 1 and 3, but can also phosphorylates nuclear proteins such as histone H3 and HMG-14 [259,261], through which regulates a wide range of cellular responses including inflammation, cell cycle, autophagy [262] apoptosis, cell differentiation and senescence [261]. Furthermore, p38 MAPK is involved in the pro-inflammatory response in the brain, released from microglia and astrocytes and it has also functions in glutamate excitotoxicity [263] and in pathological AD related processes [264]. Increased p38 MAPK activity in human brains from AD patients was observed, it occurs at the early stage of the disease and it is associated with neuritic Aβ plaques and NFT [265,266]. High levels of phosphorylated p38 (p-p38) is found in almost every AD pathological lesion, showing positive correlation between activated p38 and the level of aggregated tau, given that p-p38 may be involved in the tau hyperphosphorylation [267]. Furthermore, in vitro and in vivo studies have been reported that, p38 MAPK activation due to Aβ, results in increased intracellular calcium, ROS production and mitochondrial stress that lead to neuronal cell death [268–270]. Moreover, Aβ is a trigger for microglial release of inflammatory mediators, especially IL-1β activating neuronal p38 MAPK cascades [271]. Microglial p38

MAPK also contributes to the inflammation of the AD brain, it colocalized with activated microglial cells, and the inflammatory reaction is accompanied by cholinergic hypofunction [272]. In microglia p38 MAPK pathway contributes to the proinflammatory cytokines production and release [273], while in neurons it drives to tau phosphorylation, Aβ plaque and NFT formation and depression of synaptic plasticity [264,274]. Given that, inhibiting p38 MAPK activity may alleviate inflammatory diseases and neuroinflammation, by decreasing pro-inflammatory cytokines production [275]. At this purpose p38 MAPK inhibitors as neuroprotective agents have been explored. Noteworthy, the p38 MAPK inhibitor 069A, upon oral administration, dampened the Aβ-mediated IL-1β and TNFα release in mouse hippocampus and significantly improved behavioral deficit [276]. Recently, selective p38α MAPK inhibitors have shown promising results in animal models, in which ameliorate Aβ-induced synaptic and cognitive dysfunction and spatial memory deficits [277–279], and prevent cognitive impairment reducing tau hyperphosphorylation [280]. Therefore, p38 MAPK inhibitors represent an emerging therapeutic approach for the treatment of neurodegeneration and more research is required. Nevertheless, given the complex nature of AD pathophysiology, a single kinase inhibitor might not provide therapeutic efficacy [275].

2.2 PI3K/AKT signaling pathway

Phosphoinositide 3-kinases (PI3K)/Akt pathway is an intracellular signaling pathway of great importance in the cell cycle process and it is associated with cellular quiescence, proliferation, cancer and longevity. PI3K exists in multiple isoforms which have been divided into three classes (class I, class II and class III), based on structural features and lipid substrate preference [281]. Several signaling proteins, including protein serine-threonine kinases and growth factors result in PI3K activation. When PI3K is activated by tyrosine kinases and receptors associated with G proteins (GPCR), it is brought to the membrane and generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), which recruits a subset of PIP3 effectors, including exchange factors for GTP-binding protein (GRP-1), adaptor proteins (GAB-1) and proteins with pleckstrin homology (PH) domains, such as AKT and PDK1 [282,283].

- AKT

AKT, also known as protein kinase B (PKB) is a serine/threonine kinase belongs to the cAMP-dependent AGC (PKA, PKG, and PKC) protein kinase super family [284]. Three AKT/PKB isoforms have been identified PKBα (AKT1), PKBβ (AKT2) e PKBγ (AKT3) which have a highly conserved domain structure: a PH domain, a central kinase domain and the typical hydrophobic motif of AGC kinases [285]. AKT is activated following the phosphorylation of two key regulatory sites: Thr308 in the activation loop within the kinase domain and Ser473 in the C-terminal regulatory domain [284]. Phosphorylated AKT (p-AKT) fulfills various biological functions, including cell metabolism, proliferation, transcriptional regulation, and neuronal cells survival

through activation of a wide range of downstream substrates among them Bad, forkhead box protein O (FOXO), glycogen synthase kinase 3β (GSK3β) and mammalian target of rapamycin (mTOR) [286,287].
GSK-3β is an important AKT downstream molecule that is involved in neuroprotection and stimulates cell survival through cell proliferation and inhibition of apoptosis [288,289]. Moreover, PI3K/AKT/GSK-3β pathway regulates Wnt/β-catenin signaling pathway, that plays a pivotal role in neuronal function, morphology, neurogenesis and in the maintenance of synaptic plasticity [290]. Abnormal expression of these pathways is linked to neurodegenerative disorders, in particular it appears to be crucial in AD because it promotes Tau protein hyper-phosphorylation in neurons and in glial cells [291,292], memory impairment and increased Aβ production [293]. However, Aβ triggers GSK-3β which induces NFT formation by tau hyper-phosphorylation [294]. The reduction in GSK3β abnormal activation, has been reported to dampen not only Aβ accumulation, but also tau phosphorylation and neurodegeneration in AD mouse model [295].
Besides, dysregulation of the PI3K/AKT/mTOR pathway is reported in AD brains. The kinase mammalian target of rapamycin (mTOR) is a major modulator of autophagy characterized by two multiprotein complexes: mTOR complex 1 (mTORC1), a negative autophagy regulator, and mTOR complex 2 (mTORC2), required for AKT phosphorylation and activation [296]. mTOR regulates both protein synthesis and degradation, and hence brain functions linked to learning and memory [297,298].
Hyperactivation of the PI3K/AKT/mTOR pathway is linked to disrupted Aβ and tau, synaptic loss and cognitive decline in AD [299]. Increased AKT activation and mTOR signaling, have been described in hippocampal and cortical neurons in AD brains [300] and it is associated with an increase in Aβ levels, due to the autophagy impairment [301]. Therefore, inhibiting mTOR activity by rapamycin may rescue cognitive impairments and delay AD progression, alleviating both Aβ and tau protein misfolding problems [302,303]. However, conflicting results have been reported about the use of mTOR inhibitors as therapeutic strategy, since loss of mTOR signaling, can lead to detrimental outcomes, impairing long-term potentiation and synaptic plasticity [304]. In addiction has been reported that rapamycin treatment, increases Aβ generation by reducing the α-secretase ADAM-10 activity [305,306]. Given that, PI3K/AKT/mTOR pathway activation is crucial for synaptic activity and normal function. Thus, a useful pharmacological strategy should protect neuronal tissue stimulating the normal PI3K/AKT/mTOR survival pathway and promoting constitutive autophagy.

2.3 cAMP response element binding protein - CREB

CREB is a transcription factor that regulates many cellular responses including long-term memory, cell survival and proliferation, apoptosis, differentiation [307]. CREB also plays many different roles in immune function promoting anti-inflammatory immune responses, through the inhibition of NF-kB activity and the induction of anti-inflammatory cytokines as IL-10 [308]. Several protein kinases including ERK1/2 and

PI3K/AKT signalling pathways, upstream regulate CREB activation [220], which is induced by growth factors, neurotransmitters, and stress signals that promote CREB phosphorylation at Ser133. Activated CREB binds to the cAMP-response element of target gene promoter regions and induces gene expression, through the recruitment of transcriptional cofactors [309,310]. In addiction CREB plays a major role in memory acquisition and consolidation [243], promoting the long-term potentiation (LTP) and enhancing memory functions through the transcription of genes involved in synaptic plasticity and long-term memory [311,312]. Thus, dysfunctions at this pathway result in memory and cognitive impairment, reported in neurodegenerative diseases. Decreased levels of total and phosphorylated CREB (p-CREB) and cAMP have been found in the prefrontal cortex and in the hippocampus of postmortem AD brains [313,314], suggesting that CREB signaling pathway is detrimental in AD and is involved in the disease pathogenesis. Moreover, p-CREB expression is found to be reduced in peripheral blood mononuclear cells (PBMC) from AD patients and it correlated with low p-CREB levels observed in postmortem brains [314]. Thus, p-CREB levels in periphery could be an indicator of CREB signaling pathway in the brain and might be useful as a biomarker of cognitive function in AD [315].

Given that, a therapeutic strategy that can lead to cAMP elevation and, in turn increase p-CREB could be helpful to rescue the memory impairment. At this purpose, phosphodiesterase type 4 (PDE4) inhibitors which restore the cAMP/PKA/CREB signaling pathway have been tested in animal models showing rescues in LTP, improvement in synaptic plasticity and memory function [316,317]. However, CREB is involved in a wide range of physiological processes in many tissues and it is also involved in oncogenesis [318]. Chronically increasing p-CREB levels could present critical drawbacks, therefore CREB itself may not to be the best molecular target for therapy [319].

2.4 p70 S6 kinase

P70S6 kinase together with p85S6 kinase, represent mitogen-activated serine/threonine kinases which constitute the ribosomal S6 kinase 1. These two isoforms are generated from the same transcript by two different translation start sites and present different localization, while 70S6K is mainly cytoplasmic, p85S6K isoform is exclusively nuclear [320]. p70s6k is involved in regulation of cell-cycle, cell differentiation, and motility, it is a downstream effector of the PI3K and ERK1/2 signaling pathway [321], and its activity is controlled by mTOR, a key autophagy regulator [306,322]. Abnormal p70S6K activation has been associated with hyperphosphorylated tau formation and NFT accumulation in AD patients [323], in particular has been reported that p70S6K phosphorylation at the Thr421/Ser424 is directly correlated with t-tau and p-tau levels [323]. p70S6K can also phosphorylate tau at Ser262, Ser214, and Tht212 sites, which lead to tau release from microtubules, resulting in microtubule disruption [324]. In addiction in the lymphocytes of AD patients have been found a significant decreasing in phosphorylated p70S6K, and it is correlated with MMSE scores, suggesting that the decline of cognition in AD patients corresponds to the decrease in

p70S6k levels [325,326]. Moreover, Aβ has been shown to lead to disruption in PI3K/AKT/mTOR/p70S6K signaling that results in deregulation of the autophagy and apoptosis which contribute to neuronal degeneration [327,328].

2.5 Autophagy

Autophagy is a highly regulated cellular process that mediates the degradation of intracellular components, macromolecules and organelles inside lysosomes. [329]. Autophagic process is activated as response including nutrients deprivation, bacterial infection, aggregated and misfolded proteins, and damaged organelles [329]. Under physiological conditions, it plays an important role in multiple biological processes, including metabolism, cellular development and differentiation, regulation of innate and adaptive immunity. Moreover, autophagy protects against genome instability and necrosis, preventing cancer and neurodegeneration [330,331]. Three major forms of autophagy have been described: micro-autophagy, macroautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy is the major pathway for organelle and protein turnover, which are packaged into double-membraned autophagosomes and delivered to lysosomes for degradation and recycling of amino acids [332,333]. Conversely, in micro-autophagy, lysosomes directly take up cytosolic components through invagination of the lysosomal membrane [333]. Degradation by CMA involves the selective delivery of cytoplasmic proteins across the lysosomal membrane upon the recognition and binding of a pentapeptide KFERQ motif on chaperone proteins that are recognized by the cytosolic tail of lysosomal associated membrane protein 2A (LAMP2A), resulting in their unfolding and degradation [334].

Autophagosome formation is mainly governed by autophagy-related (Atg) proteins and by two protein complexes: the class III PI3K/Vps34 which forms a complex with Beclin-1, and the serine/threonine protein kinases ULK1 and ULK2 which are positive regulator of autophagosome formation [335]. The activity of the ULK complex is negatively regulated by mTOR and it is positively regulated by the AMP activated protein kinase (AMPK). In nutrients-rich situation, mTORC1 binds to the ULK1 complex inhibiting autophagy, whereas during starvation mTORC1 dissociates from the ULK1, triggering the activation of a multiprotein complex containing Beclin-1 and class III PI3K/Vps34 which regulate the autophagosome formation [330,331]. Another ubiquitin-like system involved in auto-phagosome formation is the processing of microtubule-associated protein light chain 3 (LC3B). Upon autophagy induction LC3B, is proteolytically cleaved by Atg4 to generate LC3B-I which is activated by Atg7 and then transferred to Atg3 followed by conjugation to phosphatidylethanolamine to generate processed LC3B-II, recruited onto the growing phagophore [333,336]. In addiction LC3B-II has been proposed to act as a receptor for selective substrate such as the polyubiquitin-binding protein p62/SQSTM1, a common component of protein aggregates that is found in a wide range of protein aggregation diseases [337]. Moreover, the p62-LC3 complex is involved in linking

polyubiquitinated protein aggregates to the autophagic machinery, facilitating the clearance of aggregate-prone proteins [338].

Autophagy can play either a cytoprotective or cytopathic role in different pathophysiologic processes. Actually, dysregulation of the autophagic response has been described in various pathophysiological situations, including neurodegeneration, aging, cancer, cardiovascular, infectious and inflammatory diseases [331].

- Autophagy in Alzheimer's Disease

Autophagic process is essential for homeostasis and development of the CNS. Autophagy plays a pivotal role in neuronal degradation of expired organelles, and in abnormal intracytoplasmic contents clearing which otherwise could lead to protein accumulation and neuronal activity impairment [339]. Thus, deficiency or disfunctions of autophagic process can result in neuronal degeneration characterized by intraneuronal proteins accumulation [340]. Moreover, lysosomal system inactivation is responsible for the accumulation of autophagosomes observed in AD [341]. Macroautophagy plays an important role in turning over APP and clearing Aβ peptide under physiological conditions, whereas is demonstrated to be involved in Aβ generation under pathological condition or in aging process [342]. Immature autophagic vacuoles of all components of the Aβ formation system, including APP and β-/γ-secretases, have been found accumulated in dystrophic neuritis from AD brains, thus could be a source of Aβ generation [343,344]. In addition, the impairment of the autophagic activity could result in an imbalance Aβ species, leading an increase in the $A\beta_{42}/A\beta_{40}$ ratio which is related to the production of toxic Aβ oligomers [345]. Aβ in turn, has been shown to affect autophagy. Monomers cause intracellular accumulation of autophagosomes, resulting in inhibition of substrate degradation due to reduction of lysosomal activity [346]. Impaired autophagy at the level of induction or autophagosome formation points toward Beclin1, which has been shown to be significantly reduced in entorhinal cortex and hippocampus in brain of AD patients [347,348]. Beclin-1 deficiency disrupts neuronal autophagy, increasing intraneuronal Aβ accumulation, and extracellular deposition. Moreover, it also compromises microglial phagocytosis and protein shunting, contributing also to Aβ accumulation [348]. However, transcriptional data indicate that autophagy-activating factors are upregulated in AD brains, implying increased autophagic activity. Thus, may reflect a compensatory effect at the transcriptional level induced by impaired proteostasis, or that autophagy initiation is related to disease progression [349].

$A\beta_{42}$ was found hyperactivating PI3K/AKT/mTOR signaling pathway, which plays a central role in proteostasis and in autophagy regulation[299]. mTOR activation results in autophagy inhibition, decreasing in Aβ clearance and also contribute to aberrant hyperphosphorylated tau aggregation [350]. Pharmacological

inhibition of mTOR signaling with rapamycin, rescues cognitive deficits and ameliorates Aβ and Tau pathology by increasing autophagy in AD animal models [298,302], whereas many clinical trials have demonstrated an inability to reverse cognitive impairment at moderate to late stages of disease. However, rapamycin treatment also tempers the neuroimmune response, which is heavily implicated in AD pathogenesis [351].

Besides Aβ, autophagy can also regulate Tau protein clearance, dysfunctions of the autophagy-lysosome system, promote the formation of Tau oligomers and accumulation of NFT [352]. Postmortem studies have reported that hyperphosphorylated Tau immunoreactivity is associated with LC3 and p62-positive autophagosomes in the brains of AD [353].

CMA also is linked with neurodegenerative diseases, since it plays an important role in both Tau tangles and Aβ plaques generation and its activity is impaired with aging process [354]. Tau possesses two KFERQ motifs and can interact with the cytosolic Hsc70 chaperone protein which leads its binding to the CMA receptor LAMP2A. Aggregates, Tau mutant forms and fragments are translocated to the lysosomal membrane through Hsc70 recognition, where they are cleaved by CMA process, generating fragments which are linked to NFT formation [355].

The involvement of autophagy in AD is two-fold, as it contributes to the elimination of pathogenic proteins, while it is also affected by these aberrant proteins.

Restoration of autophagic processes could promote the degradation of modified, misfolded and aggregation-prone proteins, but also prevents their detrimental effects. Nevertheless, there are few reported clinical results demonstrating that modulation or induction of autophagy represents an effective therapeutic intervention [356].

- Autophagy and Inflammasome interplay

Autophagy is also involved in inflammatory immune responses, regulating several immune functions such as antigen presentation, lymphocyte development and cytokine secretion [330]. Deletion of gene encoding the autophagy protein ATG16L1, results in excessive production of interleukin 1β (IL-1β) and IL-18, released in response to inflammasome activation [357]. Inflammasomes are intracellular protein complexes formed by nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing-3 (NLRP3) sensor, apoptosis-associated speck-caspase recruit domain (ASC) adaptor and the effector pro-caspase-1, whose assembly results in inflammation. It’s activation is triggered by a wide range of stimuli, several microbes and aggregated Aβ, which lead to the maturation and the release of the pro-inflammatory cytokines IL-1β and IL-18 [358]. An abnormal inflammasome activation plays a pivotal role in autoimmune and inflammatory diseases, including AD [359].

Autophagy can negatively regulate inflammasome activation in multiple ways through the removal of inflammasome activators, clearing the inflammasomes protein components, and mediating the

downstream cytokines degradation [360]. In particular, the inflammasome adaptor ASC is subject to the lysine 63-linked ubiquitination and interacts with p62 which mediates its delivery to the autophagosome for degradation [361]. On the other hand, inhibition or autophagy defects, exacerbate inflammasome activation and result in increased pro-inflammatory cytokines release [362], leading to development of inflammatory disorders [363].

As a matter of fact, neuronal autophagosome formation and lysosomal degradation is impaired in AD. In particular, Beclin-1 results markedly decreased in the brains of AD patients and its depletion has been reported to worsen the deposition of Aβ peptides [364], which in turn trigger the inflammasome activation and its downstream mediators release [365].

Inflammasomes and autophagy can mutually regulate each other, suggesting that this two-way street regulation, allows to preserve the balance between the natural inflammatory response and the prevention of excessive inflammation, which can be harmful in many tissues and underlies many inflammatory and neurodegenerative diseases [366].

Therefore, autophagy would be a promising therapeutic target for treatment of neurodegenerative and inflammasome-related inflammatory disorders. Therapeutic agents able to enhancing autophagy or rescuing its disfunctions could alleviate inflammasome-dependent inflammation [367,368].

Chapter 3

Neuroinflammation

3. Neuroinflammation

Neuroinflammation is referred as a damaging condition of the nervous system, characterized by the activation of the immune response in the neural tissue in order to restrain infections, eliminate pathogens, cell debris, and misfolded proteins. It is an essential component of the CNS innate immunity other than a complex and precisely regulated process, which initially plays a role in the fixation and resolution of neural tissue [369]. Nevertheless, if neuroinflammatory state becomes persistent, is detrimental for neuronal cells, contributing to neurodegeneration and functional impairment. For these reasons, neuroinflammation underlies several neurological disorders, including neurodegenerative diseases [370] and their involvement in AD is suggested by the presence of inflammatory mediators in AD brains [371].

Since the brain has been considered an immune-privileged organ because of the presence of the BBB, the infiltration of peripheral immune cells in the CNS, was thought to be tightly controlled and possible after the blood-brain barrier breakdown, which is always accompanied by the production of pro-inflammatory cytokines and glial activation [372]. However, have been demonstrated that there is a close relationship between the CNS and the peripheral immune system even though the brain is structurally and functionally unique, it requires effective immune mechanisms to cope the infections [373].

In particular, different immune cell types such as astrocytes and microglia express specialized pattern recognition receptors (PRRs) that activate immediately after the injury occurs and can trigger inflammatory signalling pathways which are able to sense pathogen-associated molecular patterns (PAMPs), but also endogenous modified molecules called damage-associated molecular pattern (DAMP) that are present in infected and injured area [374]. In this process, cellular and molecular immune components such as cytokines, complement and pattern-recognition receptors are contributing players, and they can lead to glial cells activation [375]. However, these immune cells have also beneficial non-inflammatory roles in the brain, providing proper neural functions [376]. Therefore, the beneficial or detrimental effects of neuroinflammation in the brain, depend on the duration of the inflammatory response [377]. Chronic activation of an innate immune response in the CNS, which lead to excessive production of proinflammatory cytokines in the brain, can lead to behavioral deficits and promote neurotoxicity [378].

Thus, it has been reported an association between neurodegenerative disorders and neuroinflammatory events, based on the high expression of several pro-inflammatory mediators and the presence of activated complement system and microglial cells in brain regions in which neurodegeneration occurs, suggesting that neuroinflammatory responses could start before the neuronal loss [379,380].

Ageing is associated with alterations in the neuroinflammatory environment, priming microglial cells which result in an exaggerated inflammatory cytokine response. Thus, suggests the aged brain resides in

a chronic state of neuroinflammation which contribute to neurodegenerative diseases development [381,382].

3.1 Microglia

Microglial cells are the resident macrophages of the CNS, representing around 10%-15% of the CNS population and play a pivotal role, not only in neurogenesis, neuronal plasticity, and regeneration but also in immune defense [383]. They have the ability to perform phagocytosis, release cytotoxic factors and behave as antigen presenting cells [384]. Under physiological conditions, microglia exhibit highly ramified and motile cell processes that protect the brain by stimulating phagocytic clearance of extra neurons and synaptic connections, increasing the efficiency of neuronal transmission and providing trophic sustenance to preserve cerebral homeostasis [383,385].

Microglia immune activity is restrained by specific inhibitory pathways that suppress unwanted inflammatory responses and tissue destruction that are often associated with immune activation [384]. These checkpoint mechanisms include direct inhibitory interactions between microglia and neurons through the receptor-ligand pairs CX3CL1-CX3CR1 and CD200-CD200R, in order to maintain the balance of pro- and anti-inflammatory cytokines [386,387]. During infections, aging and neurodegeneration, microglia acquire an activated phenotype, morphologically characterized by a reduction in branch number and an increase in cell soma volume [381,388], therefore they rapidly move towards the lesion site, where they promote an inflammatory response releasing pro-inflammatory cytokines [389].

Microglia express many PRRs and Toll-like receptors (TLRs), such as TLR4 and TLR1/2 and NOD-like receptors (NLRs), such as the NLRP3 inflammasome that detect PAMPs or DAMPs such as bacterial molecules, neuronal ATP or aberrant endogenous proteins [390]. Moreover, microglial cells express chemokine receptors such as CCR2 and CCR4, fractalkine receptors CX3CR1 and CXCR4, as well as integrins, such as CD11b and CD11c, which control migration and positioning of microglia within the CNS [391]; they express also purinergic receptors for ATP as P2RY12 and a wide range of immune receptors that regulate microglial activation, among them TREM2 [392,393]. Microglial cells also express the trans-membrane protein 119 (TMEM119), a cell-surface protein of unknown function which is specifically expressed by parenchymal myeloid cells in the CNS and is the best promising candidate for human microglial marker [394,395].

Microglia were initially described using a dichotomous paradigm, distinguishing between a ramified static condition and an amoeboid inflamed one. The activation states of microglia was categorized into “classical pro-inflammatory activation” (M1) and “alternative anti-inflammatory phenotype” (M2) as commonly described for macrophages [396]. In particular, M1 phenotype represents pro-inflammatory activity triggered by LPS or IFN-γ, whereas in the second one, the response is induced by IL-4 or IL-13. The M1-microglia produce TNF-α, IL-1β, IL-6, NOS, hydrogen peroxide and matrix metalloproteinases, which are

helpful in host defense but can also lead to neuronal damage [397,398]. Instead, M2-microglia exhibit an anti-inflammatory phenotype by expressing IL-10 and transforming growth factor-b (TGF-β), which promote tissue remodeling and healing [398,399].
Although this characterization is useful to describe microglia activation, recent transcriptome-based studies have demonstrated that there are many other dynamic intermediate functional states, which reflect microglial phenotypes during development, homeostasis, and disease[400,401] and this huge diversity is strictly regulated by cytokines, which play a pivotal role in both physiological and pathological conditions in the brain.

The brain shows an increasing inflammatory state with aging and this altered inflammatory profile is linked to alterations of microglia functions [392]. Microglia undergoes several age-related phenotypic changes compatible with their activation, which are associated with a chronic inflammation phenotype due to an increased production of inflammatory cytokines and ROS, leading to the loss of their neuroprotective function [402]. Microglia repairing abilities become dysregulated and these cells acquire an irregular distribution and shorter cellular processes [403]. These events have been linked to a greater susceptibility to cognitive deficits and the onset of chronic neurodegenerative diseases.

- Microglia and Alzheimer's Disease

Microglia results in an acute immune response in the brain against harmful stimuli, including misfolded proteins such as Aβ, which cause neuronal stress and hypersensitization of microglia, inducing their activation to produce cytokines and neurotoxins, hence promoting neurodegeneration [404,405].
In healthy brain and early stages of AD, microglia is able to restrict senile plaque deposition by secreting proteolytic enzymes, such as neprilysin, and MMP9 and expressing receptors that promote Aβ clearance and phagocytosis, such as scavenger receptor CD36, and receptor for advanced-glycosylation end products (RAGE) [406,407], suggesting their beneficial role in preventing plaques formation. During AD pathogenesis, this phagocytic activity seems to decline, since microglia become dysfunctional because of the altered expression of proinflammatory mediators and neurotoxic molecules, leading to an impairment of Aβ clearance and processing [408]. High levels of chemokines, adhesion molecules and acute phase proteins are usually associated to activated microglial cells and result in their long-lasting activation, which produces ROS, and other neurotoxic molecules, generating a vicious cycle of neuroinflammation [409–411].
Moreover, microglia express a range of different TLR and PRRs which are able to respond to DAMPs or PAMPs and also bind to Aβ species with various affinities, triggering inflammation [412]. Engagement of these receptors induces TNF-α and IL-1β release, which mediate neuroinflammation and result in

sustained low-grade inflammation [413]. In addiction, these cytokines were found upregulated in brains of AD patients and in transgenic mice with AD-like pathology [414].

Furthermore, have been reported that microglia are also able to clear Aβ through a process termed LC3-associated endocytosis (LANDO) that is also involved in receptors such as TREM2 and TLR4, recycling. The depletion of this process in animal models, leads to increasing Aβ accumulation, microgliosis with release of proinflammatory cytokines and tau hyperphosphorylation [415]. On the other hand, phagocytosis of Aβ can trigger microglial activation. Following phagocytosis Aβ activates the NLRP3 inflammasome, leading to caspase-1 activation and IL-1β maturation and release [365].

Even though Aβ represents a trigger for microglial cells, it has also been shown to prevent plaques formation and LPS stimulation reduced amyloid deposition as well, stimulating microglia clearing properties [416]. On the other hand, these cells are also identified in NFT and they are able to influence tau phosphorylation but, in this case, LPS injection worsened the pathology [417]. Moreover, microglial cells play a direct role in the spread of pathological tau in the brain [418,419]. Given that, both Aβ and tau trigger microglial activation, they stimulate different aspects of the neuroinflammatory response, and the activation of the immune system in the brain represents a key link between the two major pathological hallmarks of AD [420].

Furthermore, it is possible visualize microglial and astroglial activation in AD patients. To date, PET exams demonstrated an increased number of activated microglial cells in AD patients, which well correlates with memory impairment [421]. Moreover, a significant correlation between MMSE and microglial activation levels has been found in cortical regions of AD patients, underlining the association between microglial activation and cognitive decline [121].

The involvement of microglia in AD pathogenesis is also demonstrated by the presence of activated microglia around amyloid plaques from AD patients [422], and more recently GWAS analysis have identified AD risk genes from microglia membrane proteins including TREM2, TYROBP, CR1 and CD33 [423]. In particular, single-nucleotide polymorphisms in CD33 and TREM2 genes have been directly linked to impaired Aβ uptake and phagocytosis [424,425]. Post-mortem brain samples of AD patients showed high expression levels of CD33 in microglia surrounding Aβ plaques and its overexpression in animal models, is lead to impaired Aβ uptake, suggesting that CD33 is associated to Aβ accumulation [424,426].

The activation of the innate immune system plays a role in AD pathophysiology. However, activated microglia might have a neuroprotective or a cytotoxic role depending on disease context.

3.2 TREM2

Triggering receptor expressed on myeloid cells 2 (TREM2) is a cell surface receptor of the immunoglobulin superfamily expressed by monocyte derived dendritic cells and microglia in the CNS [427]. In the brain, TREM2 is required for microglia migration, cytokine release, phagocytosis, lipid sensing, ApoE binding, shielding of amyloid plaques, and proliferation [428,429]. It interacts with the transmembrane region of DAP12 (TYROBP) in order to mediate signaling events through DAP12's immunoreceptor tyrosine-based activation motif (ITAM) domain [430]. When ligands bind TREM2, DAP12 is phosphorylated on the cytoplasmic tyrosine residues by Src kinases, and phosphorylated DAP12 recruits the spleen tyrosine kinase (Syk), which in turn, activates multiple downstream signaling mediators such as ERK, PI3K, phospholipase Cγ (PLCγ) [431,432]. Moreover, TREM2/DAP12 signaling triggers protein and lipid phosphorylation cascades that result in Ca2+ mobilization, integrin activation, cytoskeleton rearrangement and MAPK signaling [433]. In addiction TREM2 is crucial for maintaining microglia metabolism and autophagy [433]. During homeostasis, TREM2 mediates phagocytosis of microbial, endogenous ligands and also apoptotic neurons, to facilitate debris clearance following injury [434], while microglial activation and proinflammatory cytokines, rapidly reduce TREM2 surface expression, which undergoes sequential proteolytic processing by ectodomain shedding and intramembrane proteolysis [435]. The α-secretases ADAM17 and ADAM10 cleave TREM2 close to the His157 residue of the large extracellular domain, releasing soluble TREM2 (sTREM2) [183,435], while the remaining TREM2 c-terminal fragment (CTF) is further degraded by γ-secretase [435]. When this degradation is impaired, the accumulated TREM2 CTF can interact with the DAP12 limiting the interaction between full length TREM2 and DAP12. This results in reduced TREM2-mediated signaling, which locks microglia in a homeostatic state and inhibits their transition to disease-associated microglia (DAM), in addiction lack of functional TREM2 leads to reduced cellular proliferation, impaired phagocytosis and reduced Aβ clearance [436,437]. Furthermore, defective TREM2 signaling affect microglial energetic metabolism, mTOR activation and autophagy. Thus, TREM2-deficient microglia is not able to respond properly to stressful events, such as Aβ toxicity [183].

Rare loss-of-function variants in the TREM2 gene R62H [28] R47H and H157Y, were significantly associated with increased AD risk. These variants dramatically reduces TREM2 expression, compromising myeloid cell responses to amyloid pathology [438]. The AD risk mutations probably alter TREM2 function by impairing ligand binding and as a result, TREM2 signaling [439]. Postmortem brain specimens from AD patients carrying R47H genetic variant, have shown a decreased microglial response to Aβ plaques and increased neural dystrophy, suggesting that R47H is a partial loss-of-function variant that affects microglia activation and proliferation in response to Aβ plaques [429,440]. TREM2 deletion in amyloidosis models has been reported, to alter phosphorylation of tau protein, which leads to enhanced intraneuronal tau phosphorylation, aggregation, and kinase dysregulation [441]. In contrast, in human tau transgenic mouse model, TREM2 deletion had a protective effect, preventing tau-driven synaptic loss and atrophy in the hippocampus and

entorhinal cortex [442]. Other AD risk variants, such as R62H and R62C, impair phospholipids binding in vitro, but the impact of these rare TREM2 mutations in vivo remains to be established [443].
Given that, TREM2 has a protective role for microglia in AD pathogenesis, whereas TREM2 loss of function worse AD progression. Thus, the boost in myeloid cell function by pharmacological modulation of TREM2 activity could be helpful in neurodegenerative diseases such as AD. At this purpose, monoclonal antibodies raised against TREM2 were screened and tested, in order to boost TREM2 expression and signaling, preventing its shedding and bolsters microglial survival and also Aβ phagocytosis [184,185]. Noteworthy, the anti-human TREM2 agonistic monoclonal antibody AL002c, has been shown promising results in a first-in-human phase I clinical trial [444].
TREM2 promotes the optimal microglial function required to attenuate AD progression and, a potential target for eliciting a protective role for microglia in AD and other neurodegenerative diseases [445,446]. Although several studies suggest that targeting TREM2 could decrease AD-related pathologies, whether a potential TREM2-targeting treatment will be effective in sporadic AD patients, which represent the majority of cases it is still unknown.

3.3 NLRP3 Inflammasomes

Inflammasomes are multiprotein complexes, mainly expressed in myeloid cells, activated upon cellular infection or stress and formed by the assembly of multiple subunits that regulate the maturation and the secretion of pro-inflammatory cytokines to engage innate immune defenses [358].
The (NOD)-like receptor protein 3 (NLRP3) inflammasome is currently the most fully characterized inflammasome which is activated in response to several pathogens, as well as PAMPs and DAMPs [447].
NLRP3 is a 115 kDa cytosolic protein composed of three domains: a C-terminal leucin-rich repeat (LRR) with regulatory functions, a central nucleotide-binding and oligomerization domain (NACHT) or nucleotide binding oligomerization domain (NOD) which have ATPase activity, and a N-terminal pyrin domain (CARD) or pyrin domain (PYD) that recruits ASC [448,449] and triggers its helical fibrillar assembly. ASC fibrils then recruit the effector caspase-1 via CARD interaction, leading the formation of ASC “speck” complex [450,451]. Thus, acts as a molecular platform that recruits pro-caspase 1, which is then activated by autocatalysis and induces the post-transcriptional processing and the subsequent release of pro-inflammatory cytokines such as IL-1β and IL-18 [452,453]. This pathway is defined for “canonical” inflammasome form, whereas the “non-canonical” inflammasomes are activated by cytosolic LPS and result in caspase 4, caspase 5 and caspase 11 activation [454].
In addition, all inflammatory activated caspases, trigger a pro-inflammatory form of cell death called pyroptosis. This process is mediated by the cleavage of the cytoplasmic gasdermin-D (GSDMD) protein,

which leads to the release of its N-terminal domain that oligomerizes as pores in the cells plasma membrane by which mature IL-1β, IL-18 and IL-1α are released [455,456].

Activation of the inflammasome requires two tightly regulated and interconnected pathways: a priming and an activation step. In the brain, the priming step is provided by the activation of an innate immune sensor or cytokine receptor, which trigger the myeloid differentiation primary response protein (MyD88)-NF-κB pathway leading to the generation of additional inflammatory mediators such as pro-IL-1β [457,458]. Activation signals are provided by different stressors including changes in plasma membrane permeability, lysosomal damage, and metabolic stress [459]. ATP is a NLRP3 agonist and stimulate inflammasome activation triggering the P2X7R receptor, inducing substantial changes in K^+ efflux, which lead to inflammasome activation [460]. Moreover, crystalline substances and aggregated misfolded proteins, induce lysosomal damage and cell stress, leading to mitochondrial disruption, which in turn trigger inflammasomes activation [461]. In particular, Aβ oligomers and fibrils can activate inflammasome acting as DAMP. Thus, NLRP3 activation may represent a critical component of the inflammatory response in Alzheimer's disease [365].

Therefore, the NLRP3 inflammasome plays a crucial role in the development of inflammatory responses in the CNS. Moreover, emerging studies have demonstrated the involvement of NLRP3 signaling in several neurological disorders, including multiple sclerosis, amyotrophic lateral sclerosis, prion diseases, and AD.

- NLRP3 and Alzheimer's disease

Activation of microglia and the subsequent innate immune response have been identified as key hallmark in AD, which contribute to disease pathogenesis and progression [462]. Several studies have focused on mutual interaction of innate immune mechanisms and neurodegenerative processes. In particular, the NLRP3 inflammasomes expressed by myeloid cells and neurons, play a key role in mediating the innate immune reaction in AD [463].

Aβ represents the main DAMP in AD patients' brains and can activate NLRP3 inflammasome through different mechanisms. NLRP3 inflammasome is activated by different size of Aβ aggregates and also by lower molecular weight Aβ oligomers and protofibrils, suggesting that microglial activation by these Aβ species may trigger the innate immune responses in the CNS before the onset of Aβ deposition [464].

The inflammasome activation due to Aβ deposition, is mediated by the formation of ASC specks, which represent the adaptor proteins in the NLRP3 complexes. Moreover, ASC specks complexes have been found in activated microglia and in the extracellular space [452]. Microglial cells undergo pyroptosis upon strong NLRP3 activation and release ASC specks which bind to Aβ peptides, thereby increasing their aggregation [465]. Moreover, ASC-Aβ complexes have been identified in brain samples from APP/PS1 mice and AD patients [466], these aggregates result in amplified NLRP3 inflammasome activation leading to pyroptotic cell death. In addition, extracellular ASC aggregates are associated to impaired Aβ-clearance

by microglia [467]. Furthermore, Aβ oligomers can interfere with neuronal membrane functions and evoke potassium efflux from neurons which is a potent activator for the inflammasomes, and in turn stimulate caspase-1 to cleave pro-IL-1β and pro-IL-18 cytokines [468]. A further evidence associating the activation of inflammasomes with AD is the high activation of caspase-1 in patients' brains. Caspase-1 activity has been reported strongly increased in brains from MCI and early-onset AD patients, suggesting that NLRP3 inflammasome activation may represents an early pathogenic event [466]. Thereby, high levels of the pro-inflammatory cytokine IL-1β are detected in microglial cells surrounding Aβ plaques in AD patients' brains and CSF [469]. Additionally, elevated levels of IL-1β in AD patients promote the activation of p38 MAPK signaling, leading to tau hyperphosphorylation [271]. Inhibition of caspase-1 activity, has been reported protecting from spatial memory impairment, loss of synaptic plasticity, associated behavioral disturbances and other consequences related to AD [470]. Moreover, have been also observed that peripheral monocytes derived from AD patients, co-express NLRP3, ASC and caspase-1 and showed increased IL-1β and IL-18 release [359]. The downstream effectors of NLRP3, can further induce the activation of glial cells in the CNS and the subsequent release of inflammatory molecules, creating a feedback loop to increase the production of pro-inflammatory mediators which lead to NFT and Aβ plaques formation [471], affecting synaptic plasticity and inhibiting long-term potentiation, which subsequently impair learning and memory [472].

Although the Aβ-induced NLRP3 inflammation has been shown to be crucial for the development and progression of AD, the link between NLRP3 activation and Tau hyperphosphorylation is still poorly elucidated.

Recently have been reported that also Tau aggregates can activate NLRP3 inflammasome in ASC-dependent manner, following their microglial uptake and lysosomal processing [473]. Moreover, NLRP3 inhibition or loss of function, reduced tau hyperphosphorylation and aggregation by regulating Tau kinases and phosphatases in an IL-1β-dependent manner [474]. Thus, suggest that NFT may develop downstream of Aβ-induced microglial activation and provide a link between Tau pathology and NLRP3 activation.

An additional mechanism by which NLRP3 activation can contribute to AD pathogenesis is in response to dying neurons releasing ATP, which binds to the purinergic receptor P2X7R on microglia [475]. Activated P2X7R induce a decreasing in intracellular K^+ that activate NLRP3 and consequently exacerbate inflammation and damage [476]. Furthermore, preclinical and clinical studies have been reported an upregulation of P2X7R in microglia from AD patients [475].

Given that, the microglia-specific activation of the NLRP3 inflammasome is pivotal for AD pathogenesis and contribute to amyloidosis and neuropathology. Targeting NLRP3 components, such as the receptor, caspase-1 or ASC, could prevent inflammasome assembly and reduce pro-inflammatory cytokines release. Thus, it could be a promising approach to cope neuroinflammation and slow AD progression. At this

purpose, have been also demonstrated the NLRP3 inhibition or caspase-1 knockout, could largely protect from memory loss and decrease Aβ deposition in an AD mouse model [466], suggesting the higher possibility of targeting NLRP3 inflammasome in AD therapy.

- Targeting NLRP3 inflammasomes

In a wide range of preclinical models have been demonstrated that inhibition or deficiency of NLRP3 inflammasome components or downstream products, might provide beneficial effects in inflammatory and neuroinflammatory diseases. Hence, there is substantial interest in the discovery of potentially therapeutic inflammasome inhibitors. Exogenous compounds that may block or inhibit NLRP3 inflammasome dependent cytokines and IL-1β signaling, are already approved for therapeutic use [477,478]; however, targeting IL-1β expression could not be the best therapeutic approach in AD. IL-1β is not the only product of NLRP3 inflammasome activation and many others proinflammatory mediators are involved in AD pathogenesis [479]. Whereas, identifying a direct blockade of inflammasome activation instead of targeting its downstream cytokines, could be an effective strategy. A few small-molecule compounds have shown anti-inflammatory effects on NLRP3 inflammasome activation in vitro and in AD animal models. JC-124, a selective NLRP3 inhibitor, has been reported to suppress the NLRP3 inflammasomes assembly and the activation of caspase-1, dampening the production of IL-1β, which results in reduced amyloid deposition and alleviated AD-associated deficits in AD mouse model [480]. Other compounds of interest are the fenamate class of non-steroidal anti-inflammatory drugs (NSAIDs), which can inhibit the NLRP3 inflammasome ameliorating the cognitive impairments in rodent models of AD [481]. Glyburide is a hypoglycaemic drug used for type II diabetes mellitus treatment. This compound has been reported, to affect NLRP3 inflammasome activation preventing ASC oligomerization and acting downstream of the P2X7 receptor, impeding the inflammasome activation by DAMPs, and crystalline substances. However, Glyburide is known to have some several side effects and it could not be the best compound to target NLRP3-related inflammation [482,483].
The best characterized small-molecule inhibitor of the NLRP3 inflammasome is MCC950 or CRID3, a diarylsulfonylurea-containing compound. This synthetic molecule directly and specifically inhibits both canonical and noncanonical NLRP3 inflammasome activation pathways and the subsequent IL-1β release and pyroptosis by preventing NLRP3-induced ASC oligomerization [484]. Moreover, have been shown that MCC950 inhibits inflammasome and microglia activation in AD mouse models, enhancing Aβ phagocytosis, decreasing plaques accumulation, and consequently ameliorating cognitive functions [485]. Even though the mechanism of action and the molecular target are still to identify, have been reported that MCC950 directly interacts with the Walker B motif within the NACHT domain of NLRP3, thereby blocking ATP hydrolysis [486]. Furthermore, MCC950 is also highly specific for NLRP3 and it does not inhibit

any other identified inflammasomes or TLR-driven pathways [487]. Therefore, it is the most selective tool compound available for investigation and a promising candidate for further development for the treatment of NLRP3-related pathologies.

Nucleoside reverse transcriptase inhibitors (NRTIs), including stavudine (D4T), inhibit NLRP3 inflammasome activation blocking P2X7R or a P2X7-dependent pathway [488] and prevent the transcription of proteins, including caspase-1 and, consequently the maturation of IL-18 [489]. Recently, have been reported that D4T hampers the assembly of the NLRP3 inflammasome, prevents the caspase-1 activation and stimulates Aβ autophagy by macrophages [490]. However, more extensive studies are necessary to establish whether agents targeting the NLRP3 inflammasome, could offering a therapeutic opportunity.

NLRP3 inflammasome is an attractive pharmacological target since its inhibition could hamper pathological inflammation. Nevertheless, further information are needed regarding where possible interferences with the brain's innate immune system will be beneficial [453]. Further key point is the development of drugs that cross the BBB and selectively inhibit only those inflammasomes that are involved in the particular pathogenetic processes. To date, no drugs have currently been established to directly bind and inhibit the NLRP3 inflammasome since the detailed activation mechanisms are still to elucidate [491].

Chapter 4

Aim

4.1 Intracellular signal transduction and potential drug treatment against β-amyloid cytotoxicity

Alzheimer's disease is a major public health concern and has been identified as a priority for scientific research. Although there are approved treatments that can mitigate the symptoms, there is an urgent need to improve our understanding of the pathogenesis to facilitate the development of disease-modifying treatments.

Several other hypotheses have been proposed to explain the mechanisms underlying the AD pathogenesis such as oxidative stress, alteration of intracellular homeostasis, glutamate excitotoxicity, inflammation, cholinergic systems impairment, deterioration of protein degradation systems and activation of caspases which consequently lead to neuronal apoptosis or deregulation of intracellular signal transduction pathways. However, the primary alterations that trigger the neurodegenerative process remain to be clarified.

The two core pathological hallmarks of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles. The amyloid cascade hypothesis suggests that an imbalance between the production and clearance of Aβ in the brain is the initiating event, consequently leading to neuronal degeneration, cells death and dementia [492]. In particular, soluble oligomers are the most toxic species of Aβ, which impair the normal synaptic functions underlying neuronal and synaptic dysfunction in the progression of AD. These toxic Aβ species disrupt synaptic plasticity, inhibit hippocampal LTP, induce the production of ROS and also interact with several protein kinases leading disturbances in intracellular signal transduction [52]. In addiction Aβ accumulation induces the release of inflammatory mediators by the microglia; this facilitates Aβ deposition and neuroinflammation in a self-feeding pathogenic loop[493]. During AD progression, several growth factors and mitogen compounds are modified, mediating their cellular effects through activation of PI3K/AKT and MAP-Kinase signaling [219,244]. These pathways regulate a wide range of cellular processes and biological functions, which are essential for the maintenance of cell homeostasis, survival and physiological functions; therefore in the CNS, they are also involved in the maintenance of memory and cognitive functions [494]. Furthermore, AKT and MAP-Kinases signaling upstream regulate Tau-phosphorylation and Aβ catabolism, also through autophagy, and they are involved in APP processing, promoting the non-amyloidogenic pathway and the subsequent secretion of sAPPα by α-secretase [237,241], too.

Several cellular and biochemical abnormalities that have been detected in CNS, such as oxidative stress, glutamatergic system impairment, disturbances in protein kinase activity and APP metabolism are also noticeable in peripheral fluids and tissues from AD patients, such as plasma [495] and CSF [129], monocytes [496], platelets [497,498], and fibroblasts as well [499,500]. In particular, cultured skin fibroblasts derived from AD patients mirror events that have also been demonstrated to occur in the AD brain [501]. Since in the brain

of AD patients, MAPK and PI3K/AKT signaling modulations seem related to the alterations and deposition of Aβ and Tau, we investigated these specific pathways in primary cultures from human fibroblasts. In this way, we considered systemic but specific molecular mechanisms involved in AD, especially in the early phase of disease. Furthermore, fibroblasts are independent of the postmortem changes thus, they can be suitable for pharmacological and molecular studies [499].

In the last decade, the association between diet and cognitive function has been largely investigated. Much attention has been focused on the potential role of natural compounds as neuroprotective agents, to attenuate or suppress inflammation, oxidative stress and Aβ mediated cytotoxicity. In particular, Hop (Humulus Lupulus) contains flavonoids, aromatic molecules which have antioxidant, anti-inflammatory and anti-atherogenic properties. It has been shown that Hop extract has anti-aggregating effects on Aβ oligomers and seems to prevent its production in cultured cells [502]. In addition, a growing number of flavonoids have been shown to inhibit the development of AD-like pathology and to reverse cognitive deficits in AD animal models, suggesting that these compounds might be useful in dementia to prevent its progression and to promote cognitive performances [208,503].

- Aim of the study

Considering the findings described in the paragraph above, the aim of the first part of this study is characterize the complex interactions among Aβ and MAPK and PI3K/AKT signaling in fibroblasts from sporadic AD patients, with different disease severity, and, in order to evaluate any molecular mechanisms that could prevent or modulate Aβ-induced toxicity, we also investigated the potential protective role of Hop extracts against Aβ toxicity, and their influence on Ras/MAPK and PI3K/AKT pathways.

At this purpose we investigated the modulation of specific signal transduction pathways which are deregulated in AD. We assessed the phosphorylation state of stress-activated protein kinase (p38 and JNK), which are involved in the alteration of synaptic plasticity caused by Aβ and are linked to Tau hyperphosphorylation [264,504]. We analyzed the involvement of RAS-ERK and PI3K-mTOR pathways assessing the activation rate of PI3K/AKT and ERK1/2 signaling pathways. Then, we compared p-ERK and p-AKT status with AD and MCI MMSE, in order to identify a possible correlation to the disease severity.

Furthermore, we evaluated some their downstream pathways, Bax and p70S6K, which regulate apoptosis, autophagy, and Tau synthesis and hyperphosphorylation, in order to better investigate the regulation of Tau metabolism. Since ERK1/2 signaling pathway modulates APP cleavage, we also assessed cytosolic αAPP levels in fibroblasts.

These pathways may also play a central role for disease progression, and they might be important targets for therapeutic strategies. Therefore, we assessed the antioxidant and anti-inflammatory properties of

hop extracts and their involvement in Ras/MAPK and PI3K/AKT pathways, in order to evaluate any molecular mechanisms that could prevent or modulate Aβ-induced toxicity.

4.2 Investigation of the involvement of NLRP3 inflammasome activation on Ras/MAPK and PI3K/AKT pathways and autophagy modulation.

In addition to phagocytosis, it has recently been shown that the degradation of extracellular Aβ fibrils by microglia is also dependent on autophagic processes, and that autophagy is also important for the regulation of Aβ-mediated NLRP3 inflammasome activation [505]. Alterations of autophagy, a finely regulated intracellular process that mediates lysosomal degradation of proteins and organelles has also been involved in the pathogenesis of AD. Interestingly, a decrease in the activity of the autophagic-lysosomal pathway has been reported during aging in every tissue, included neurons [506]. Moreover, have been largely reported that neuroinflammation can downregulate autophagy pathway, [505,507,508] and the PI3K/AKT and ERK1/2 kinases seem to be involved in its modulation, as well as in Aβ and Tau processing and in immune-inflammation pathways [244,471]. Autophagy is a complex molecular system that mediates the degradation of intracellular components and proteins from the cytoplasm and is involved in apoptosis and neurodegeneration [330]. Three main forms of autophagy have been described: macroautophagy mTOR-Beclin-1 dependent, microautophagy endosomal mediated, and CMA which is associated with lysosomal degradation and it is mediated chaperone proteins, which interacts with the channel protein LAMP2A [509].

Furthermore, recent results showed that nucleoside reverse transcriptase inhibitors (NRTI), including stavudine, inhibit the activation of the inflammasome [488], suggesting that these compounds could be helpful in AD treatment. NRTIs hamper NLRP3 inflammasome activation blocking the purinergic receptor P2X7R [488] and prevent the transcription of proteins, including caspase-1 and the subsequent maturation of IL-18 [489].

Besides extracellular Aβ plaque deposition and intracellular neurofibrillary tangles aggregation, several studies have reported a strong contribution of neuroinflammation and microglial response to AD pathogenesis.

Peripheral monocytes and macrophages are recruited as well as in the CNS of AD patients, possibly in an attempt to counteract the formation or the extension of Aβ plaques [510]. The initiation of the inflammatory response by microglia and peripheral immune cells involves cytosolic multiprotein platforms known as inflammasomes [511]. Activation of the intracellular inflammasome complex and the consequent production of high levels of proinflammatory mediators, is suspected to play a pivotal role in AD-associated neuroinflammation [359]. The activation of NLRP3 inflammasome complex by Aβ in microglia results in formation of ASC protein complexes (ASC specks), and IL-1β and IL-18 release, which subsequently induce

Aβ aggregation, contributing to the progression of the pathology and aggravation of cognitive alterations [465]. The possible role of inflammasome activation in AD is supported by data indicating that Aβ can activate the NLRP3 inflammasome and that NLRP3 inflammasome deficiency facilitates the differentiation of microglia cells to an M2 (anti-inflammatory) phenotype, resulting in a decreased deposition of Aβ in the APP/PSI mouse model of AD [466,512]. The interference with the activation of this pathway represents an attractive alternative strategy for AD therapy.

- Aim of the study

Considering the findings described in the paragraph above we investigated the involvement of NLRP3 inflammasome activation on Ras/MAPK and PI3K/AKT pathways and autophagy modulation, in $A\beta_{42}$ treated peripheral blood immune cells of AD patients. Our investigation was focused on ERK1/2, PI3K/AKT, p38MAP-Kinase signaling pathway and their downstream pathways Beclin-1, LAMP2A, p70S6K and Bax, which are involved in autophagy and apoptosis regulation respectively. We also investigated the effects of the nucleoside reverse transcriptase inhibitor Stavudine, that reduces NLRP3 inflammasome activation, on autophagy modulation. Furthermore, were also analysed possible D4T-induced modulations of CREB, this transcription factor is involved in immune responses, inducing macrophage survival and proliferation and regulation of T and B lymphocytes. CREB plays an essential role in promoting the anti-inflammatory immune response, through the inhibition of NF-kB activity and the induction of IL-10 [308,513]. Moreover, CREB plays a key role in memory acquisition and consolidation, promoting the LTP and neuronal plasticity [243].

The bi-directional communication existing between brain and periphery provides the possibility to study central alterations at a systemic level. Thus, patients with different disease severity and healthy controls were selected for blood samples withdrawal in order to isolate PBMCs to perform our analyses. Monocytes were then isolated from PBMCs, which provide a peripheral model of central myeloid cells precursor of microglia, in which high level NLRP3 inflammasome expression were detected in AD.

In addition, THP-1 cells differentiated with PMA in macrophage-like cells were used to perform preliminary investigations of D4T effects on Ras/MAPK and PI3K/AKT pathways, autophagy modulation and inflammasome activation.

THP-1 macrophage-like cell line as well as PBMC-derived monocytes from AD patients and healthy controls were primed with LPS and stimulated with Aβ in presence or absence of D4T. Aβ oligomers and fibrils can activate inflammasome acting as DAMP [365] and this peptide has a central role in AD pathology. Thus, we were interested in verifying if D4T could counteract the disturbances on intracellular signaling pathways and inflammasome activation due to Aβ oligomers, in acute treatments and in patients.

Furthermore, we were also interested in analyzing the interplay between autophagy and NLRP3 inflammasome activation. Autophagy can downregulate inflammasome activation, through the removal of its activators, clearing its protein components such as ASC, and mediating the downstream cytokines degradation [360].

Based on the fact that D4T can hamper the NLRP3 inflammasome activation and, as a consequence, neuroinflammation we are interested in investigation of D4T molecular mechanisms and to better understand whether it could be useful in AD.

4.3 Investigation of NLRP3 inflammasome pathway activation in Aβ-related pathology

In the last part of this study, we focused our attention on the role NLRP3-inflammasome activation pathway by Aβ oligomers in AD pathogenesis. In fact, AD is associated with neuroinflammation, but it is not clear yet whether this process is a reaction to Aβ accumulation in the CNS, or if inflammation is an attempt to increase Aβ clearing. The events which trigger inflammation as well as the mechanisms associated with its intensity and maintenance are still unclear. The over production of Aβ oligomers in the brain, can result in the increased of Aβ phagocytosis by the microglia and by the recruited peripheral monocytes. This could lead to lysosome damage which can be an endogenous signal for inflammasome activation. As well as for cell signaling studies, the test experiments were performed in THP-1 cells, to set up the best experimental conditions in order to analyze NLRP3 inflammasome activation, assessing pro-inflammatory cytokines release on supernatants and NLRP3-related proteins expression. In order to investigate the NLRP3 inflammasome activation pathway, monocytes derived microglia cells (MDMIs) were differentiated from monocytes isolated from AD patients and HC. These cells are a versatile in vitro model of human adult microglia that may be an alternative to primary microglia for large-scale in vitro studies [514]. Monocytes were differentiated into microglia and characterized for surface and intracellular protein expression to assess whether MDMIs display a myeloid phenotype. We are interested in investigating whether NLRP3 inhibition by CRID3 treatment, a selective NLRP3 inhibitor [486], could prevent or counteract the alterations on microglial phagocytosis due to inflammasome activation. At this purpose, we evaluated key microglial functions such as pro-inflammatory cytokines release, phagocytosis and degradation upon Aβ stimulation and well characterized NLRP3 inflammasome activators [460], with or without CRID3. Compared to D4T, which inhibits NLRP3 inflammasome activation in an indirect way blocking the ATP receptor, CRID3 is a selective and direct NLRP3 inhibitor [487]. It is also our interest investigate the effects of inflammasome inhibition through these two different pathways.

Thus, the overall goal is to characterize inflammasome activation pathway in MDMIs in order to better understand if the dampening of the inflammasome assembly and activation, could be an attractive beneficial strategy for AD therapy.

Chapter 5

Materials and Methods

5.1 Population study

Twenty AD patients (six severe, eight moderate and six mild), 7 MCI subjects and 13 age-related controls took part into the study. Their socio-demographic and clinical features are shown in Table 1 [515]. Criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) were applied for the diagnosis of probable AD or MCI [103]. All patients presented with progressive cognitive impairment predominantly affecting memory and were assessed with brain Computed Tomography (CT) or nuclear magnetic resonance imaging (NMRI), blood tests for excluding secondary dementia (thyroid hormones, folic acid, vitamin B12, VDRL- TPHA and erythrocyte sedimentation rate) and a comprehensive neuropsychological battery. AD patients were considered severe if they had a MMSE score < 12, moderate for a score between 13 and 19 and mild for a score between 20 and 24.

Exclusion criteria:

The subjects with the following features were excluded from the study:

- major depression disorders, mania and schizophrenia,
- a past or present history of substance abuse,
- neoplastic or haematologic disorders,
- recent infections or surgery,
- severe hepatic or renal insufficiency,
- myocardial infarction or head injury within the last six months

The subjects that were following a pharmacological therapy with these drugs were excluded as well, even though they did not fulfil any of the criteria above mentioned:

- antiplatelet
- anti-inflammatory
- anti-neoplastic
- corticosteroid
- immunosuppressive

No patient had a familial history of dementia. For HC, active medical illness, personal or family history of neurological or psychiatric disorders and alcoholism or drug abuse were ruled out by clinical interview. Absence of cognitive impairment was defined by a MMSE score > 26/30. Experiments were undertaken with written consent of each participant and the study was approved by the local Ethics Committee of San Gerardo Hospital, Monza, Italy

Subjects	Number	Men/Women	Age±SD	MMSE±SD
AD	20	9/11	74.2±1.99	16.63±5.09
HC	13	6/7	69.4±1.63	29.22±0.47
MCI	7	4/3	74.6±2.04	27.64±1.03

Table 1: Demographic characteristics and neuropsychological score of tested subjects. APO-Eε4 total distribution in AD (20) and MCI (7) is 11/27. For Age, One-way analysis of variance: $p=0.35$; for MMSE, AD vs HC $p< 0.01$ and $p<0.05$ vs MCI, and HC vs MCI $p>0.05$.

- Skin biopsy and primary cell culture

From HC and MCI subjects and AD patients skin biopsies were performed and cultured into Dulbecco's Modified Eagle's Medium (DMEM, Euroclone, Italy), supplemented with 10% heat inactivated Fetal Calf Serum (FCS, Euroclone), 2 mM L-glutamine, 100 U/ml penicillin (EuroClone) and 100 mg/ml streptomycin (1% P/S). The obtained fibroblasts were plated in dish and they were expanded until they reached the sub-confluence. Then they were collected in cold phosphate-buffer saline (PBS) within 12th trypsin treatment, as previously reported [516]. To obtain dry pellets, fibroblasts were centrifuged at 250xg at 4°C for 10 min, then they were preserved at -20°C, until the protein extraction.
Primary fibroblasts were seeded in petri dish 60 x 15 mm (Corning), cultured in DMEM supplemented with 10% FCS and 1% P/S and grown to confluence. Fibroblast obtained from MCI and AD patients were treated with 0,1mg/ml Hop extracts for 24h. Thereafter, cells were harvested to obtain dry pellets, and centrifuged at 250xg at 4°C for 10 min, then they were preserved at -20°C, until the protein extraction.

- SH-SY5Y - Human neuroblastoma cells

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium-F12 (EuroClone) supplemented with 10% fetal FCS, 1% P/S and 2mM L-glutamine (EuroClone), at 37 °C in an atmosphere of 5% CO_2 in air. Cells were exposed to 0,1 mg/ml Hop extracts for 30 min, 2h and 24h. Thereafter, cells were harvested to obtain dry pellets, and centrifuged at 250xg at 4°C for 10 min, then they were preserved at -20°C, until the protein extraction.

- Hop extract

Hops (Humulus Lupulus) extracts were obtained at the BioNMR laboratory of the Department of Biotechnology and Biosciences of the University of Milan Bicocca.

- $A\beta_{1-42}$ oligomers preparation

According to the manufacturer's instructions 200 µg of freeze-dried $A\beta_{1-42}$ (PM= 4514.13 g/mol) (Phoenix Pharmaceuticals) were resuspended in cold 1M Tris-Base pH 9 to obtain a 221.5 µM $A\beta_{1-42}$ solution.

- Viability assay

Cells were exposed to 0,1 mg/ml Hop extracts and 5µM Aβ oligomers. Cell viability was evaluated by MTT assay. Cells were incubated with 0.5 mg/ml MTT in standard medium for 45 min at 37 °C in an atmosphere of 5% CO2 in air. After the cells has been dissolved with DMSO, absorbance was quantified (wavelength 570 nm) using a multi-mode microplate reader (FLUOstar Omega, BMG LABTECH) and cell viability was expressed as percentage.

- Total- and phosphor-ELISA for ERK, AKT and p70S6K

In order to detect and quantify the levels of AKT (total/phosphor), ERK1/2 (total/phosphor) p70S6K (total/phosphor) in protein lysates, we used the Immunoassay Kits (InstantOne ELISA kit Invitrogen), according to manufacturer's procedure. Cytosol proteins extraction were performed in Cell Extraction Buffer (Biosource), containing 1mM PMSF, protease and phosphatase inhibitor cocktail (Sigma-Aldrich, 1:200 and 1:100), for 30min, on ice. Then lysates were centrifuged at 12000xg for 10 minutes at 4°C. According to the BioSource assay method, different dilutions of samples were tested for each phosphorylated or total protein detection. The protein absorbance was determined by plate reading at 450nm (BioRad). The concentrations were calculated comparing the tested absorbance to the specific standard curve values for each kinase phosphorylated status, and they were expressed with respect to specific kinases total status.

5.2 Population study and cellular models

- THP-1 human monocytes

THP-1 human monocytes (IZSLER, Istituto Zooprofilattico Sperimentale della Lombardia e Dell'Emilia Romagna, IT) were grown in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, and 1% P/S (Invitrogen Ltd, Paisley, UK). To differentiate these cells into macrophages, monocytes were seeded in 6-well plates at a density of $1x10^6$ cells/well and incubated for 12 h at 37°C in 5% CO2 in medium that contained 50 ηM of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO). Cells were then resuspended in serum-free medium before stimulation

- Peripheral blood mononuclear cells (PBMC) isolation

Peripheral blood mononuclear cells (PBMC) were separated from whole blood withdrawn from nine healthy control subjects and nine AD patients, who fulfilled inclusion criteria for a clinical diagnosis of AD were randomly selected within a large database of patients consecutively admitted between January 2017 and September 2019 by the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico in Milano, Italy (Table 2).

The clinical diagnosis of AD was performed according to the NINCDS-ADRDA work group criteria and the DMS IV–R. Neuropsychological evaluation and psychometric assessment were performed with a Neuropsychological Battery that included MiniMental State Examination (MMSE). The study conformed to the ethical principles of the Helsinki Declaration. All patients (or their legal guardians) gave their written informed consent for this research before entering the study.

Blood sample collection and processing and PBMC stimulation were carried out at Laboratory of Molecular Medicine and Biotechnology, Fondazione Don C. Gnocchi ONLUS IRCCS.

	Number	Gender	Age ($\bar{x}\pm\sigma$)	MMSE ($\bar{x}\pm\sigma$)
AD	9	M=2 F=7	77,2±7,2	19,8±5,0
HC	9	M=1 F=8	74,5±2,5	29,2±1,1

Table 2: Demographic characteristics and neuropsychological score of tested subjects. MMSE cut off score <26/30.

- Cell cultures

PBMC were resuspended in RPMI 1640 (PAN-Biotech GmbH, Am Gewerbepark, DE) supplemented with 10% human serum, L-glutamine (2 mM), and 1% penicillin (Invitrogen, Ltd, Paisley, UK) in 6-wells plate and incubated at 37 °C in a humidified 5% CO2 atmosphere for 2 hours in a 12 wells plate for monocyte adhesion. After 2 hours, non-adhering PBMC were harvested and discarded and monocytes grown on before stimulation. Monocytes ($7x10^6$/well) were either cultured in medium alone (Basal) or were primed with Lipopolysaccharide (LPS) (1µg/ml) (Sigma-Aldrich, St. Louis, MO) for 2 hours and stimulated with $Aβ_{42}$ (10µg/ml Sigma-Aldrich, St. Louis, MO) for 22 hours in the absence or presence of 50 µM Stavudine (D4t) (Sigma-Aldrich)

- Proteins extraction

Sub-confluent cell cultures were collected in cold PBS and lysed in Cell Extraction Buffer (BioSource) containing 1mM PMSF, protease and phosphatase inhibitor cocktail (Sigma-Aldrich) (1:200 and 1:100). After incubation for 30min on ice, lysates were centrifuged at 12,000xg for 10 minutes at 4°C. Protein concentration was determined by Bradford assay at 595nm.

- Western Blot

Proteins of cytosol (25 ug) were separated by electrophoresis on 4-12% NuPAGE® Bis-Tris gels (Invitrogen) and blotted on nitrocellulose filter (GE Healthcare). Blots were blocked 1 h at room temperature on a shaker in 5% no-fat dried milk in TBS-T buffer (50 mM Tris-HCl pH 7.6, 200 mM NaCl, 0.1% Tween 20). Blots were incubated overnight, on a shaker, at 4°C with the following Antibodies: primary rabbit anti-pERK1/2 [pTpY185/187] (1:500 polyclonal, Cell Signaling Technology Inc, Danvers, MA, USA) and anti-pAKT (1:500, monoclonal, Invitrogen); anti-pp38 [pTpY180/182] (1:350 Cell-Signaling), anti-Beclin (1:300, Cell Signaling), anti-LAMP2A (1:400, Abcam), anti-phosphorylated p70 S6-Kinases [T389] (1:300, Cell Signaling), anti-pCREB (1:800, Biosource) and mouse anti-Bax (1:300, Chemicon).
Antibodies were diluted in 5% fat- free dried milk in TBS-T buffer. A mouse anti-β-actin Ab (1:20000, Sigma-Aldrich) was used as internal standard. A peroxidase-linked anti-rabbit/mouse (1:5,000; SigmaAldrich) IgG secondary Ab was incubated for 1 hour, at room temperature on an orbital shaker in TBS-T buffer containing 3% fat-free dried milk. Signals were detected by chemiluminescent reagents (ECL Plus Kit; Amersham), visualized on X-ray film, quantified using ImageJ software and expressed as the ratio between the target and the actin signals.

- Real-time PCR

The RNA was extracted from 1×10^{6} PBMC using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was obtained using the VILO SuperScript VILO Kit cDNA Synthesis (Invitrogen) under the following conditions: 10 minutes at 25°C, 60 minutes at 42° and 5 minutes at 85°C. The DNA was quantified spectrophotometrically. PCR was performed in ABI Prism 7500HTSequence Detection System (Applied Biosystems) and the amplification of specific PCR products will be detected using the HOT FIREpol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne). Results will be expressed as ΔΔCt and will be presented as ratios between the target gene and the GAPDH housekeeping mRNA.
The following probes were used: NLRP3, β-arrestin 1 and GAPDH forward and reverse primers (Quiagen); EAAT1 forward primer (5'-GAAACGCTTGTGGGTGCTG-3') and reverse primer (5'-GTGGCAGAACTTGAAGAGGTCC-3')

- Statistical analysis

By Graph Pad Prism program statistical analysis was performed. All results were expressed as mean ± Standard Deviation (SD). One- way ANOVA analysis, followed by Bonferroni post-test, was used to determine the significance of differences among the values obtained in the subgroups of recruited subjects. By Pearson's test, possible correlations between protein-kinase expressions and MMSE score or αAPP levels were analyzed. By Student-t test, we evaluated the statistical analyses between patients' and HC group.

5.3 Patients recruiting

Four Alzheimer's disease patients which were subjected to blood withdrawal were enrolled at the Memory Clinic of the Center for Neurodegenerative Diseases and Gerontopsychiatry of the University Hospital Bonn prior written consent of each patient.

Patients were selected according to demographic informations:

- Genotypes
- Age
- Gender
- Clinical diagnosis

Inclusion criteria:

- Men and women
- Patient age ≥ 55 years and ≤ 85 years

Exclusion criteria:

The subjects with the following features were excluded from the study:

- Suspected or known abuse of alcohol, drugs or medicines, current or past
- Patients with peripheral inflammatory diseases in the last 3 months
- Head injuries with loss of consciousness of > than 5 minutes
- Living in a nursing home before the study starts

Sampling procedures and risks

The procedure involves injecting a needle into a vein to withdraw a blood sample. This is a minimally invasive process that does not pose any significant health risk to the patient.

- Blood sample collection and processing

Whole blood was collected in 4 vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMC) were separated on Ficoll gradient. Viable PBMC were counted using Bürker Chamber protocol and monocytes were isolated using Pan Monocyte Isolation Kit (Miltenyi Biotec [517]) according to the manufacturer's instructions. Briefly: PBMC were centrifuged after counting and pellet were resuspended with 40 µL of cold MACS (Dulbecco's Phosphate Buffered Saline (PBS) 1X + 0,5% BSA + 2mM EDTA) buffer per 10^7 total cells. Then, 10 µL of FcR Blocking Reagent per 10^7 total cells and 10 µL of Biotin-Antibody Cocktail per 10^7 total cells were added and the solution was incubated for 5' at 4°C. After incubation 30 µL of MACS buffer per 10^7 total cells and 20 µL of Anti-Biotin MicroBeads per 10^7 total cells were added and the solution was incubated for 10' at 4°C. The cell suspension was added

to the column for the magnetic cell separation. Flow-through containing unlabeled cells, represented the enriched monocytes fraction.

- Monocytes-derived microglial cells (MDMIs) differentiation

Monocytes were counted and centrifuged 400xg 5' RT. The pellet was resuspended in the right volume of RPMI-1640 Glutamax (GIBCO) + 10% fetal bovine serum (FBS) + 1% penicillin-streptomycin (P/S), supplemented with 100ng/ml recombinant human interleukin (IL)-34 (R&D Systems 5265-IL-010/CF) and 10ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, 215-GM-050/CF) and plated $1x10^6$/well in Matrigel coated 6 well plates. Fresh medium was added every 48h and after 6 days the plates were washed thoroughly to remove any unbound cells. After 14 days MDMIs were then harvested or used for functional assays the following day.
MDMIs were seeded at density of $1x10^6$/well in RPMI-1640 Glutamax supplemented with 10% FBS and 1% P/S and allowed to attach overnight. The following day medium was replaced with fresh RPMI-1640 Glutamax serum free. Cells were primed for 3h with 100 ng/ml Lipopolysaccharide (LPS)(Escherichia coli 0111:B4; Invivogen) and stimulated for 1h with 1mM ATP (Sigma-Aldrich), with or without 1 μM CRID3 (MCC950, Invivogen) or for 24h with 2,5 μM Amyloid beta peptide (Aβ) (PSL Peptide Specialty Laboratories Gmb). After the LPS priming and stimulation with relevant controls, the cell culture supernatants were collected and frozen at −80°C for further analysis and cells were harvested.

- Cellular models and tests experiments

THP-1 human monocytes were grown in RPMI-1640 Glutamax supplemented with 10% FBS and 1% P/S. Cells were cultured $5x10^6$ in T25 flask in medium alone (RPMI-1640 Glutamax serum free supplemented with 1% P/S) or primed for 3h with 100ng/ml LPS (Escherichia coli 0111:B4; Invivogen) and stimulated with 10μM and 20μM Nigericin (Invitrogen) for 1h.
To evaluate Aβ phagocytosis flow cytometric analysis was performed. THP-1 cells were cultured at density of $6x10^5$/well in 6 well plates and preincubated for 30 min with 40 μM cytochalasin D, as a negative control of phagocytosis. Therefore, cells were exposed to 0,5 μM FAM-labeled $A\beta_{1-42}$ (Peptide Specialty Laboratories GmbH) (FITC), 0,5 μM Aβ-TAMRA (Peptide Specialty Laboratories) (PE) and 1mg/ml pHRodo E. coli beads (Life Technologies) as phagocytosis positive control, for 6h, 4h, 2h and 30 minutes, in order to perform phagocytosis tests and select the best fluorescent tagged Aβ peptide. Aβ-FAM was selected for phagocytosis experiments in MDMIs.

- Protein precipitation from supernatant and lysates collection

MDMIs were seeded at a density of $1x10^6$ cells/well in 2mL serum free RPMI-1640 Glutamax in 6-well plates. After pre-stimulation with 100 ng/mL LPS, cells were treated as before mentioned. Then, supernatants were collected, centrifuged at 15.000xg for 5 min to remove cell debris and stored at -20°C for protein precipitation. For protein precipitation from the supernatant 500 mL methanol and 125 mL chloroform were added to 500 mL supernatant and centrifuged 5 min at 15.000xg at 4°C. Then the upper aqueous phase was removed, and 500 ml ice-cold methanol were added. Samples were then vortexed vigorously and centrifuged for 5 min at 15.000xg at 4°C. Supernatants were removed and pellets were dried in a vacuum dryer. The pellets were then resuspended in 20 ml 2X loading buffer (106 mM Tris-HCL, 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA (pH 8.5), 360 mM 1,4-Dithiothreit (DTT)) and 5 mg/mL Orange G. Subsequently, samples were subjected to western blot analysis.
For cell lysates collection, cells were scraped of the well plates, centrifuged at 15.000xg for 5 min and pellets were lysed using 1 X ristocetin-induced platelet agglutination (RIPA) buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium desoxycholate, 1% NP-40, and 0.1% SDS) supplemented with 1X Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology). Cell lysates and precipitated supernatants were denatured in loading buffer at 95°C and 360 rpm for 5 min in a thermo cycler.

- Proteins quantification

To determine protein concentrations of cell lysates, a bicinchoninic acid assay was performed using Pierce™ BCA Protein assay kit (Thermo Fischer Scientific) according to the manufacturer's protocol.

- Western Blot

Samples were separated on a NuPAGE 4%–12% Bis-Tris Gel (Invitrogen) in NuPAGE MES Running Buffer (NP0002). The Trans-Blot TurboTM Transfer System (Bio-Rad Laboratories) was used to blot the proteins on a 0.2 mm nitrocellulose membrane (Trans-Blot TurboTM Transfer Pack, Bio-Rad Laboratories). Thereafter, membranes were blocked with 3% fatty acid-free bovine serum albumin (BSA) (Millipore) in Tris-buffered saline supplemented with Tween-20 (TBST) (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0) for 1h at RT, followed by incubation with the primary antibodies mouse anti-NLRP3 (1:1000, AdipoGen), mouse anti-caspase-1 (1:1000, bally-1, AdipoGen), rabbit anti-ASC (1:1000; clone AL177, AdipoGen), mouse anti-α-tubulin (1:1000, Termo Fisher Scientific) overnight at 4°C. On the next day, membranes were washed three times in TBST and incubated with the respective secondary IRDye IgG (H

+ L) antibodies (1:20 000, LI-COR Biotechnology) for 1h at RT. Proteins were then visualized with the Odyssey CLx Imaging System (LI-COR Biosciences) and quantified using Image Studio (LI-COR Biosciences).

- Phenotypical characterization of MDMIs

At least 1x10^6 MDMIs were used for phenotypical characterization at FACS. Cells were harvested with 0.5% trypsin-EDTA solution then were washed with FACS buffer (PBS + 2% BSA (Sigma)). Cell were fixed with Fixation Buffer (FOXP3, Invitrogen) overnight at 4°C then washed with 1ml Permeabilization buffer (Invitrogen) and then incubated with the following antibodies: anti-CD11b (BV605, Biolegend), anti-CD45 (APC-Cy7, Biolegend), anti-CD14 (PE-Cy7, Invitrogen), anti-CD16 (BV421, Biolegend), anti-CD36 (APC, BD Pharmingen), anti-CD235a (PE-Cy7, Biolegend), anti-CD34 (PE, MACS Miltenyi Biotech), anti-CD43 (APC, Biolegend), anti-TREM2 (APC, R&D System), anti-TMEM119 (FITC, Biolegend), anti-HLA DR-DP-DQ (FITC, BD Pharmingen), anti-CD68 (PE, BD Pharmingen), anti-CCR2 (BV605, Biolegend), anti-CX3CR (FITC, Biolegend) and anti-P2YR12 (PE, Biolegend). MDMIs were stained with viability staining solution 7-amino-actinomycin D (7-AAD) (BD Pharmingen).

- Immunocytochemistry

MDMIs were seeded at a density of 1.5x10^5 cells/well in RPMI-1640 Glutamax + 10% FBS + 1% P/S in a 24-well plate containing coverslips and let adhere overnight. Cells were washed once with PBS 1X (Dulbecco) and fixed in 4% paraformaldehyde (PFA) dissolved in PBS for 15 min. For permeabilization, cells were washed three times with PBS containing 0.1% Triton X-100 (PTX) for 5 min. Thereafter, cells were blocked using 3% normal goat serum (Vector Laboratories) in PTX for 1h and were incubated with the following primary antibodies: anti-TREM2 (1: 500; clone 78, Millipore), anti-IBA1/AIF1 (1:250, Wako), anti-TMEM119 (1:500, Abcam) and anti-Pu.1 (1:40, Thermofisher Scientific) overnight. After three washing steps in PBS, the secondary antibody goat anti-rabbit alexa-fluor 488 conjugated (1:000; Invitrogen) was applied for 1h at RT followed by three washing steps in PBS. 40,6-Diamidino-20-phenylindol-dihydrochloride (DAPI) was used as a counterstain at 0.1 mg/mL for 10 min in PBS before coverslips were mounted. Confocal microscopy was carried out using the LSM800 Laser Confocal Scanning Microscope (Carl Zeiss Microscopy) with 20x and 40x objective. The final images processing was done using Fiji (ImageJ software).

- Pro-inflammatory cytokine release

NLRP3 inflammasome activation was determined by measuring the IL-1β and IL-18 secretion using the human IL-1β and IL-18 DuoSet ELISA kit (R&D Systems). MDMIs cells were treated with 1 ml medium per

well in 6-well plates. After the LPS priming and stimulation with ATP or Aβ treatment with relevant controls, the cell culture supernatants were harvested and frozen at −80°C. Supernatants were thawed once and assayed according to the manufacturer's protocol. The optical density was determined at 450 nm photometrically with a microplate reader (Infinite M200; Tecan). The concentration of cytokine released was quantified using the relevant standard curves.

- Aβ phagocytosis and degradation

To evaluate Aβ phagocytosis flow cytometric analysis was performed. MDMIs were seeded at 2.5×10^5 cells/well in 24-well plates (Corning) in RPMI-1640 Glutamax + 10% FBS + 1% P/S, 48 h before the assay. After 24h of incubation, the media was replaced with RPMI-1640 Glutamax serum free. The experiment was carried out the following day. As a negative control of phagocytosis, cells were preincubated for 30 min with 40 μM cytochalasin D (Sigma). Cells were incubated with 0,5 μM FAM-labeled $A\beta_{1-42}$ (Peptide Specialty Laboratories GmbH) (FITC) peptide or 1 mg/ml pHRodo E. coli beads (Life Technologies) as phagocytosis positive control, and with or without 1 μM CRID3 for 6h, 4h, 2h, 1h and 30 min. Then, MDMIs were collected by detaching with 0,5 mM EDTA solution for 10 min at 37°C and the EDTA was then inhibited with RPMI-1640 supplemented with 10% FBS and 1% P/S. After washing, cells were stained with 7-AAD and analyzed on BD FACSCanto II flow cytometer (Becton Dickinson and Company) and data were analysed using FlowJo software V.10 (FlowJo, LLC).
For Aβ degradation experiments, MDMIs were preincubated for 30 min with 40 μM cytochalasin D and exposed to 0,5 μM Aβ-FAM peptide for 6h, 4h, 2h, 1h and 30 min. Cells were washed three times in PBS and subsequently incubated for 17h in fresh medium with or without 1 μM CRID3. Thereafter, cells were collected and stained. Degradation of Aβ-FAM peptide was measured using the BD FACSCanto II Flow Cytometer and data were analysed using FlowJo software V.10 (FlowJo, LLC).

- Statistical analysis

Statistical analyses were performed using Graph Pad Prism 8 for Mac OS. Data are expressed throughout the study as mean ± SEM. Two-tailed Student's t-test or one-way ANOVA, followed by post-hoc test as specified, were used to assess the significance of differences between two or more groups, respectively. Statistical details are given in the respective figure legends. Values of $p<0.05$ were considered statistically significant.

Chapter 6

Results

6.1 Intracellular signal transduction and potential drug treatment against β-amyloid cytotoxicity

For the first part of this study, we recruited 20 Alzheimer's Disease patients (AD) (six severe, eight moderate and six mild), 7 MCI subjects which were diagnosed the pathology according to the NINCDS/ADRDA criteria and 13 age-related controls (HC). As described by the aim of this project, we are interested in characterizing the complex interactions among Aβ and MAPK and PI3K/AKT signaling, in sporadic AD patients with different disease severity, at this purpose we investigated the modulation of specific signal transduction pathways which are deregulated in AD [515].

- Modulation of stress-activated protein kinase (SAPK) in AD and HC fibroblasts

By western blot and phospho-ELISA assays, the phosphorylation state of p38 and JNK was assessed in fibroblasts from AD, MCI and age-matched HC. p38 MAPK is involved in the pro-inflammatory response in the brain and it is also associated to pathological AD related processes [266].

Results shown that p-p38 and p-JNK-SAPK protein levels were increased in fibroblasts from AD patients (Fig 1A and 1C) since the early stages of the disease, compared to HC ($p<0.05$). Comparable results were observed by phospho-ELISA ($p<0.05$) (Fig. 1B and 1D). They suggest that these modifications are probably a response to cell bioenergetic impairment. There was no correlation between p-JNK or p-p38 and disease severity.

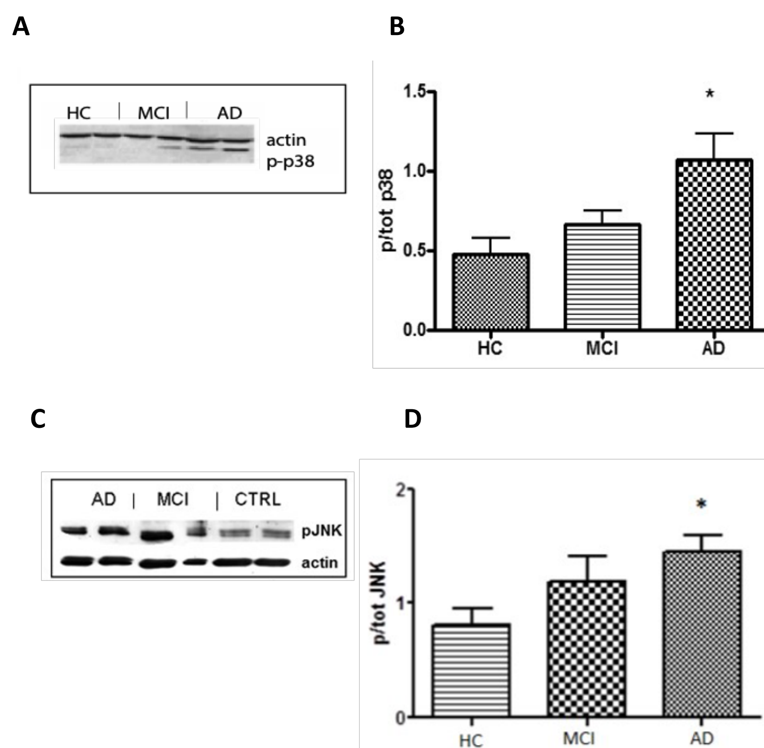


Fig 1A-D: Representative images of immunoblots in fibroblasts from AD patients, MCI and HC (**A**: p-p38, **C**: p-JNK1/2). Signals were normalized to β-actin expression (42 KDa). **C-D**: p-p38 (**C**) and p-JNK1/2 (**D**) phosphorylation status quantification by phospho-ELISA in AD, MCI and HC fibroblasts. Signals were normalized to total-p38 and total-JNK1/2. One-way ANOVA followed by Bonferroni post-hoc test *p<0.05 vs HC.

- Modulation of Ras-ERK and PI3K/AKT-mTOR signaling pathways and disease severity

We analyzed the involvement of Ras-ERK and PI3K-mTOR pathways assessing the activation rate of PI3K/AKT and ERK1/2 signaling pathways. Then, we compared p-ERK and p-AKT levels with AD and MCI MMSE, in order to identify a possible correlation to the disease severity.

Data herein show a significant increase of p-ERK levels in fibroblasts from severe AD patients compared to mild and moderate AD patients (p<0.01), while MCI fibroblasts shown decreased p-ERK with respect to HC (p<0.05) (Fig. 2A-B). No difference was observed between severe AD and HC (Fig 2A-B). A significant inverse correlation between p-ERK and MMSE score in AD patients and converter MCI was also observed (Fig. 2C), indicating a direct correlation with the disease severity. Instead in non-converter-MCI the correlation between p-ERK levels and MMSE score was lost.

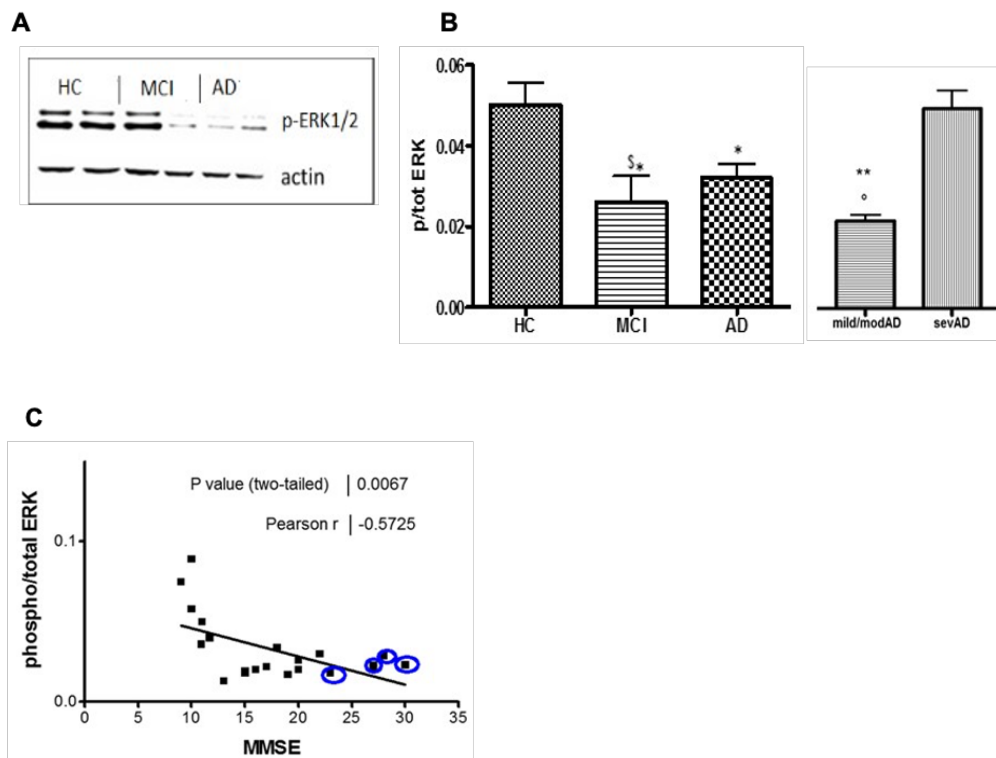


Figure 2A: A representative images of immunoblots of ERK protein levels in fibroblasts from patients and HC. Similar results were obtained by normalizing p-ERK to both tot-ERK and β-actin expression (42kDa). **B:** p-ERK levels in HC, MCI and AD patients, by phospho-ELISA, One-way ANOVA followed by Bonferroni post-hoc test *p<0.05 for MCI and AD vs HC, on the right, p-ERK levels are shown in AD patients divided according to disease severity. $p<0.05 in MCI and **p<0.01 in mild/moderate AD vs severe AD, °p<0.001 in mild/moderate AD vs HC. **C:** Negative correlation between MMSE score and p-ERK levels in AD patients and converter MCI (circles).

As ERK1/2, AKT is involved in cell survival and upstream regulates the mTOR-p70S6K signaling pathway, modulating autophagy and Tau protein phosphorylation [298]. Data herein show that p-AKT increased in AD patients compared to HC ($p<0.05$) (Fig. 3A), whereas p-AKT status was reduced comparing MCI to severe AD ($p<0.001$) and mild/moderate patients ($p<0.01$) (Fig. 3B). Moreover, we found a positive correlation between AKT phosphorylation levels and disease severity evaluated by MMSE score (Fig. 3C).

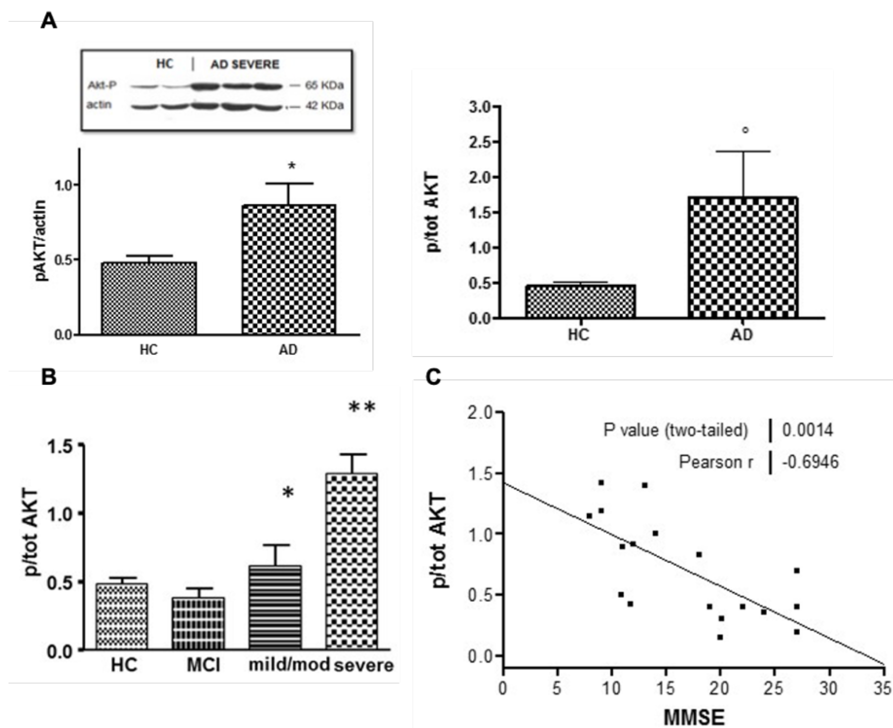


Figure 3A: A representative image of immunoblot of p-AKT protein levels in fibroblasts from AD and HC (in the box), t-Student test *p<0.05 vs HC (to the left, data expressed as pAKT/β-actin ratio). P-AKT levels by phospho-Elisa (on the right), Student's t-test °p<0.01 in AD vs HC fibroblasts. **B:** p-AKT levels by phospho-ELISA in HC, MCI and AD patients, classified according to disease severity; experiments carried out in triplicates. One-way ANOVA followed by Bonferroni post-hoc test *p<0.01 vs severe AD, **p<0.001 vs HC and MCI. **C:** The increase of p/tot-AKT levels is direct correlated to disease severity in patients' fibroblasts.

We also tested the phosphorylation of p70S6-kinase that is regulated by ERK1/2 and AKT pathway and, its phosphorylation at threonine 389 has been correlated with autophagy inhibition [327]. In fibroblasts, our results showed an increasing of phospho-p70S6K in AD patients compared to HC ($p<0.001$) (Fig. 4A). Since, Bax is also ERK1/2 and AKT downstream pathway, its expression level was detected in a subgroup of patients and HC. The cytoplasmic levels of Bax increased in AD compared to HC ($p<0.05$) (Fig. 4B), suggesting the triggering of the apoptotic mitochondrial pathway [518].

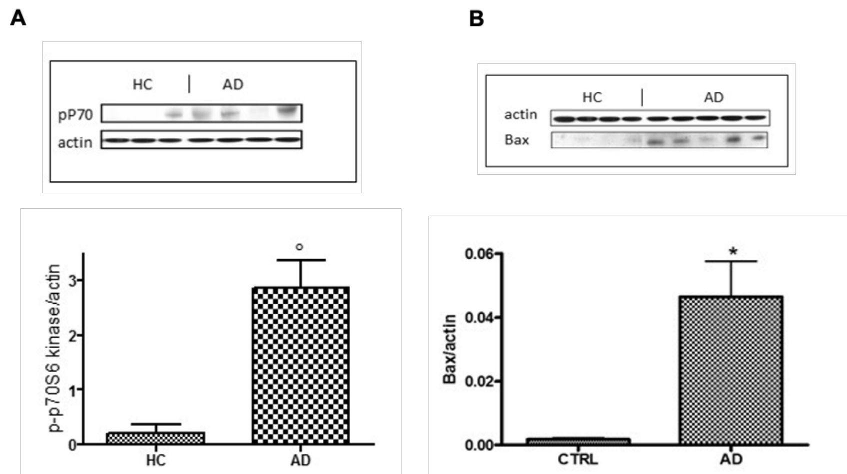


Figure 4A: A representative image of immunoblot of phospho-p70S6-kinase expression (70 kDa) in fibroblasts from AD vs HC. β-actin expression is the internal loading control (42 kDa). In histogram, the expression level was calculated as ratio between p-p70S6K and actin optical density, Student's t-test °p<0.001 vs HC. **B:** A representative image of immunoblot of Bax expression (21 kDa) in AD vs HC *p<0.005 vs HC. Actin expression is internal standard (42 kDa) data expressed as ratio of their O.D. value.

- Correlation between ERK1/2 signaling and APP metabolism

ERK1/2 is linked to APP metabolism and promotes the non-amyloidogenic APP processing by α-secretase and also negatively modulates BACE1 expression and activity, leading to reduced Aβ generation [241]. Given that, we tested the cytosolic αAPP levels in fibroblasts from recruited subjects. A significant reduction of the αAPP levels were observed in AD patients, in particular in fibroblasts from severe patients, compared to HC ones (Fig.5A). Nevertheless, p-ERK and αAPP correlation was very significant in HC, whereas in MCI subjects and AD patients this correlation was lost (Fig 5B).

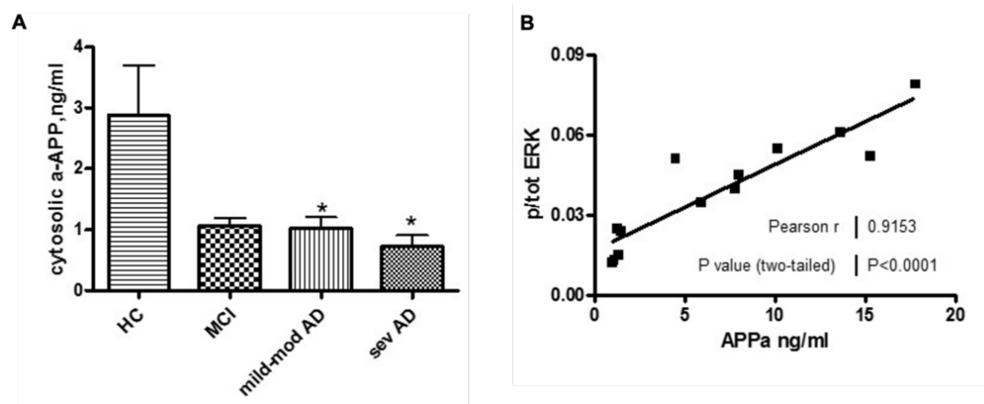


Figure 5A: Decreasing α-APP levels in MCI and AD. ANOVA followed by Post-hoc test: *p<0.05 vs HC. **B:** A representative image of the positive correlation between α-APP levels and p-ERK in fibroblasts from HC. No correlation was found in AD and MCI.

- Potential role of natural extracts against Aβ cytotoxicity

In the last decade much attention has been focused on the potential role of natural compounds as neuroprotective agents, to attenuate or suppress inflammation, oxidative stress and Aβ-mediated cytotoxicity. In particular, our attention is focused on hop extracts (Humulus Lupulus) which have antioxidant, anti-inflammatory and anti-atherogenic properties [204] and also anti-aggregating effects on Aβ oligomers and seems to prevent its production in cultured cells [502].
Given that, we assessed the antioxidant and anti-inflammatory properties of hop extracts and their involvement in Ras/MAPK and PI3K/AKT pathways, in order to evaluate any molecular mechanisms that could prevent or modulate Aβ-induced toxicity.
Human neuroblastoma SH-SY5Y cells have been used for in vitro experiments as a neuronal-like cells model. SH-SY5Y were exposed to 0,1mg/ml Hop extract (HT) for 30 min and 2h in order to assess a quick effect of this compound on kinases phosphorylation status, while we treated SH-SY5Y with HT for 24h to investigate its impact on intracellular signal transduction after longer time of exposure. Thereafter, we analysed the activation rate of PI3K/AKT and ERK1/2 signaling pathways as well as p70S6K, their downstream target.
Data herein show that HT short-term treatment (30 min and 2h) reduced AKT ($p<0.01$) (Fig.6A), ERK ($p<0.02$) (Fig.6B) and p70S6K ($p<0.001$) (Fig.6C) phosphorylation. Percentage of P-ERK and p-p70S6K is still decreased after 24h HT exposure ($p<0,02$; $p<0.05$), while no modulations are detected in p-AKT levels.

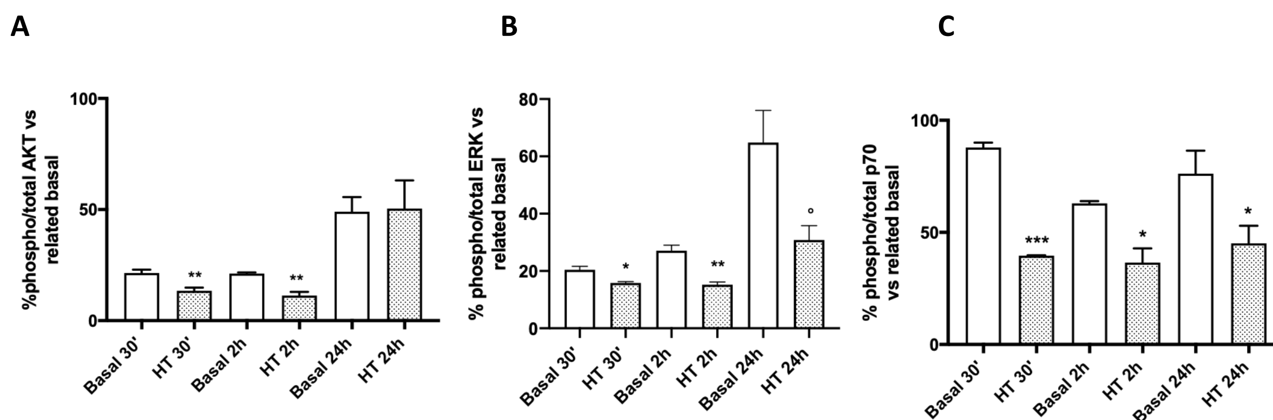


Figure 6A-C: Percentage of p-AKT, p-ERK and p-p70S6K levels by phospho-ELISA. **A**: Percentage of p-AKT levels. Student's t-test **$p<0.01$ vs related basal. **B**: Percentage of p-ERK levels. Student's t-test *$p<0.05$; °$p<0.02$; **$p<0.01$ vs related basal **C**: Percentage of p-p70S6K levels Student's t-test *$p<0.05$; ***$p<0.001$ vs related basal; experiments carried out in triplicates.

Furthermore, we evaluated the potential protective role of hop extracts against Aβ alterations on fibroblasts obtained from HC, MCI and AD patients. Previous data obtained in our laboratory have shown

that the treatment with 5 μM Aβ oligomers for 2 hours is able to induce biomolecular modifications as observed in fibroblasts from AD patients [516]. Thus, fibroblasts from HC subjects were exposed to 5 μM Aβ oligomers and 0,1 mg/ml HT for 2h, thereafter mitochondrial activity was analysed using the MTT assay in order to understand whether HT was bio-compatible, and it could counteract the Aβ induced cytotoxicity. Data herein show a decrease in mitochondrial activity of human fibroblasts following Aβ exposure compared to untreated basal (p<0.001), whereas HT treatment does not modify the mitochondrial activity and it seems to prevent Aβ toxic effect, enhancing the mitochondrial activity in Aβ co-treated cells (p<0.0001 vs Aβ) (Fig.7).

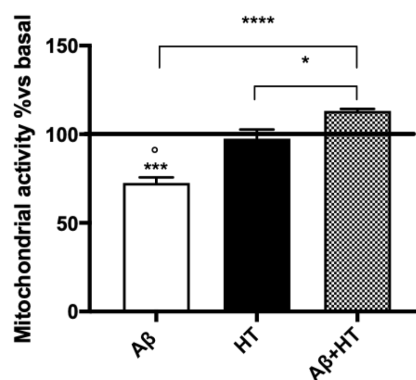


Figure 7: Effects of hop extract (HT) on Aβ-induced cytotoxicity, by MTT assay. Experiments carried out in triplicates in HC fibroblasts. One-way ANOVA ****p<0.0001 vs Aβ+HT, ***p<0.001 vs basal *p<0.05 °p<0.01 vs HT.

Given that, we investigated whether hop could modulate specific signaling pathways activated in response to cytotoxic stimuli in AD and MCI fibroblasts. At this purpose, fibroblasts from recruited subjects were exposed to 0,1mg/ml HT for 24h, in order to investigate HT involvement in PI3K/AKT and MAPK signaling pathways. In particular we focused our attention on p38-SAPK, involved in stress-induced cell signaling, and on kinases involved in cell survival and anti-inflammatory pathways, such as ERK1/2 and AKT.

Our previous data showed that p-p38 increased in MCI and AD fibroblasts (Fig.1A-D), leading to the activation of intracellular pathways that regulate the response to cellular stress. HT treatment is able to reduce pp38 levels in AD fibroblasts (p<0.02), whereas any significant modulation has been detected in MCI (Fig. 8A).

We observed that p-ERK decrease in mild/moderate AD fibroblasts (Fig. 2B), while it increased compared to untreated cells following hop treatment (p<0.01) (Fig 8B). In MCI fibroblasts p-ERK is reduced following HT exposure (p<0.05) (Fig. 8B).

Moreover, p-AKT increased in AD fibroblasts (Fig. 3A), but HT treatment was able to reduce p-AKT level in MCI fibroblasts only (p<0.02) (Fig. 8C).

Data herein suggest that Hop extract modulates specific signaling pathways activated in response to cytotoxic stimuli. This modulation might be due to Hop interaction with Aβ or its anti-inflammatory activity.

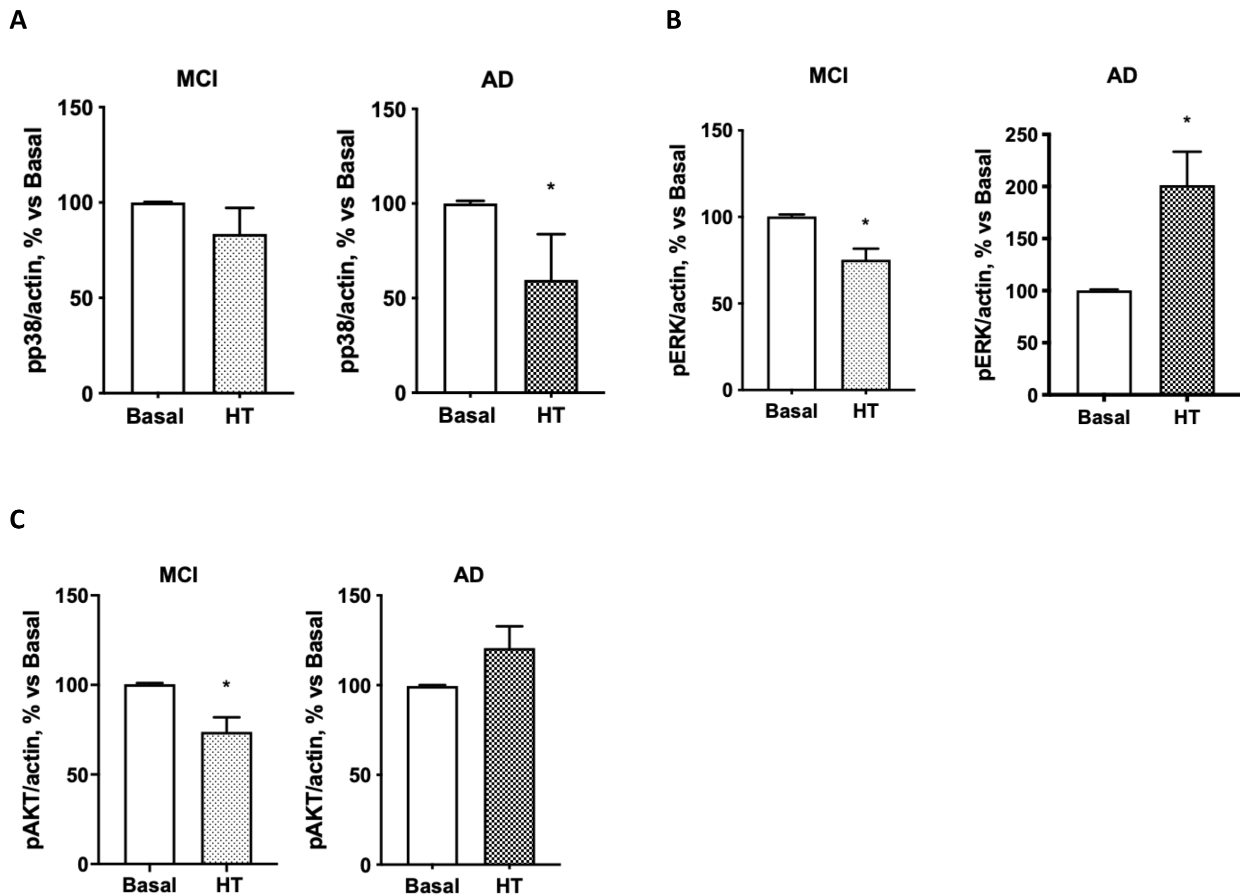


Figure 8A-C: Representative images of immunoblots of p-p38, p-ERK and p-AKT expression in fibroblasts from MCI and AD. β-actin expression is the internal loading control (42 kDa). **A:** Data expressed as percentage of p-p38/actin ratio vs basal; Student's t-test *$p<0.02$ vs basal AD. **B:** Data expressed as percentage of p-ERK/actin ratio vs basal; Student's t-test *$p<0.05$ vs basal MCI *$p<0.01$ vs basal AD. **C:** Data expressed as percentage of p-AKT/actin ratio vs basal; Student's t-test *$p<0.02$ vs basal MCI.

6.2 Investigation of the involvement of NLRP3 inflammasome activation on Ras/MAPK and PI3K/AKT pathways and autophagy modulation

In the second part of this study, we focused our attention on mechanisms involved in neuroinflammation. In particular, the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome plays a pivotal role in AD-associated neuroinflammation [359,466]. To clarify the possible involvement of inflammasome activation in Aβ phagocytosis and the potential therapeutic role of stavudine in AD, we investigated the involvement of NLRP3 activation on Ras/MAPK and PI3K/AKT signaling pathways and autophagy modulation upon D4T treatment in "*in vitro*" macrophage-derived THP-1 human monocytes cell lines and

in monocytes isolated from peripheral blood mononuclear cells (PBMC) obtained from recruited AD patients and HC subjects.

In collaboration to neuro-immunology research unit at IRCCS Don Gnocchi, we demonstrated that D4T did not increase Aβ phagocytosis in our in THP-1 cells in vitro model, analyzing the fraction of phagocyted Aβ and TREM2 mRNA expression following cytotoxic stimuli in presence or absence of D4T (data not shown) [490].
Given that, we investigated its possible involvement in modulating autophagy, an alternative phagocytic pathway. At this purpose we analyzed the phosphorylation status of ERK1/2 and AKT as well as the expression of their autophagy-related downstream targets Beclin-1, Lamp,2A and p70S6K in LPS primed and Aβ in stimulated cells for 24h with or without D4T.
We observed p-ERK and p-AKT down-regulation following D4T treatment compared to untreated cells ($p<0.05$) (Fig. 9A). These modulations might upregulate autophagy, associated with the mTOR signaling pathway and also CMA. Noteworthy the expression of autophagy-related downstream targets Beclin-1, Lamp2A, and p70-S6K, was increased by 20-to-70% upon D4T treatment compared to Aβ stimulated or LPS primed cells (Fig. 9B). Downregulation of ERK and AKT phosphorylation promotes mTOR autophagy; Beclin-1, Lamp2A, and p70S6K production results in the stimulation of autophagy as well.

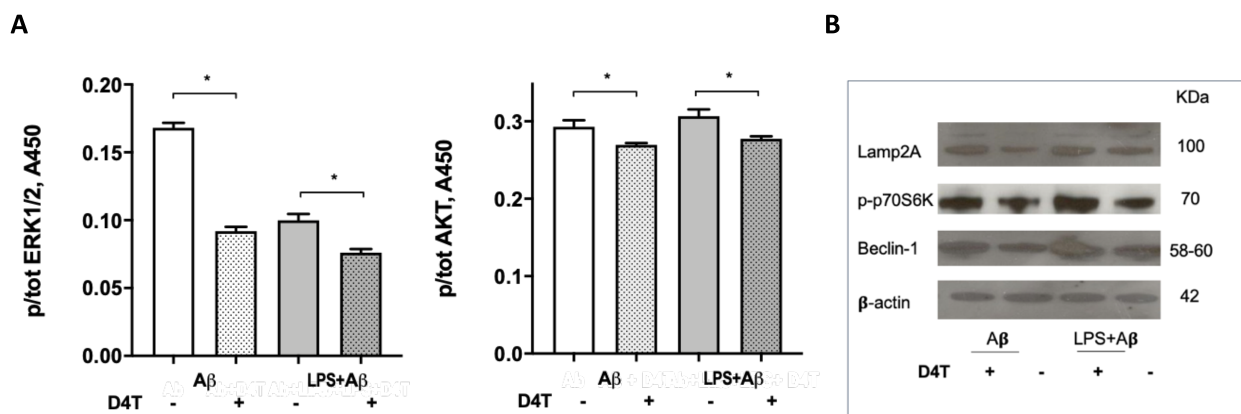


Figure 9A: Representative image of p-ERK1/2 and p-AKT phosphorylation status in cytosol protein extracts of THP-1-derived macrophages (*$p<0.05$). Results expressed as means±SD of phospho/total specific protein kinase. **B:** Representative image of immunoblots of Lamp2A, p-p70S6K, and Beclin-1. Signals were normalized to β-actin expression (42 kDa).

- D4T modulation of NLRP3, β-arrestin-1 and EAAT1 mRNA expression

By qPCR we analyzed whether D4T modulates the gene expression of the NLRP3, β-arrestin-1 and EAAT1. In order to verify whether D4T modulates the expression of the NLRP3-inflammasome qPCR analysis were performed. A significant upregulation of the mRNA expression of NLRP3 inflammasome was detected upon Aβ+LPS stimulation, suggesting inflammasome activation ($p<0.02$), whereas D4T significantly reduced NLRP3 mRNA expression ($p<0.05$) (Fig. 10A). In order to understand how D4T dampened NLRP3 activation, we analysed β-arrestin-1 gene expression. β-arrestin-1 is a member of multifunctional small molecular proteins which played a key role in the assembly and activation of NLRP3 inflammasome in the condition of inflammatory stimulation [519,520]. Data herein show that β-arrestin-1 gene expression is increased following LPS priming and Aβ stimulation ($p<0.01$), while D4T treatment reduced its expression ($p<0.05$) (Fig. 10B), suggesting a role of D4T in dampening inflammasome activation by decreasing β-arrestin-1.

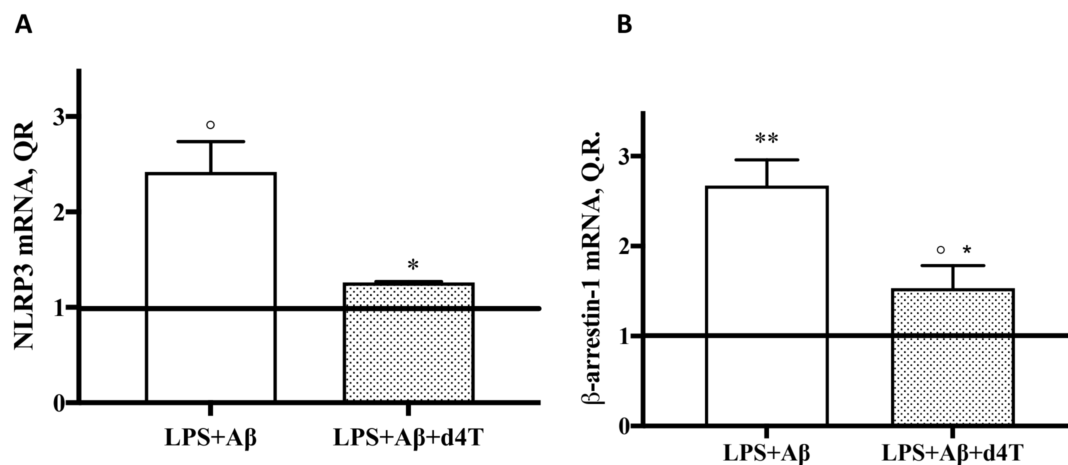


Figure 10: NLRP3 (**A**) and β-arrestin-1 (**B**) mRNA expression calculated relative to GAPDH housekeeping gene in THP-1-derived macrophages that were either unstimulated (medium) or were stimulated with Aβ after LPS priming in the presence/absence of D4T. Data are expressed as mean±SEM. **A:** °$p<0.02$ vs Basal *$p<0.05$ vs LPS+Aβ. **B:** **$p<0.01$ vs basal *$p<0.02$ vs basal °$p<0.05$ vs LPS+Aβ one-way ANOVA

The glutamate transporter excitatory amino acid transporter 1 (EAAT1) is mainly expressed in glial cells in the CNS, but also in peripheral cells [521]. It is upstream regulated by the ERK/CREB pathway through phosphorylation and its expression is impaired in AD patients, increasing the susceptibility to glutamate toxicity and contributing to neuronal damage [95,500]. Considering the D4T modulation observed in ERK1/2 signaling pathway, we evaluated the gene expression of EAAT1. In THP-1 derived macrophages, a significant upregulation of the EAAT1 mRNA expression was detected upon LPS+Aβ stimulation ($p<0.05$), even though, D4T did not modulate the transcription of the glutamate transporter (Fig.11).

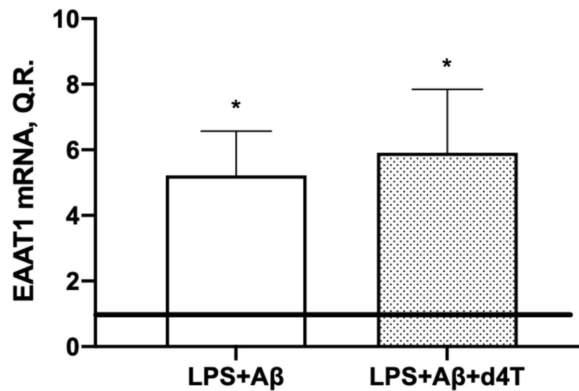


Figure 11: EAAT1 mRNA expression by THP-1 cells. EAAT1 mRNA expression calculated relative to GAPDH housekeeping gene in THP-1-derived macrophages that were either unstimulated (medium) or were stimulated with Aβ after LPS priming in the presence/absence of D4T. Data are expressed as mean±SEM. * $p<0.05$ vs Basal one-way ANOVA

- D4T modulation of Ras/MAPK and GSK3β/PI3K signaling pathways

ERK, p38 and AKT phosphorylation levels were evaluated by western blot in PBMC-derived monocytes from HC and AD patients.

In our "in vitro" model, D4T had not any effect on Aβ-phagocytosis, but it stimulated autophagy-mediated Aβ-clearing [490], as evidenced in our results obtained in THP-1 cells, D4T modulated ERK1/2 and AKT signaling pathway and upregulated LAMP2A and p70S6K, their downstream autophagy-related targets.

Since the molecular and biochemical mechanisms involved in AD have been detected also at systemic level, the analysis of intracellular signaling pathways in ex vivo peripheral models can provide useful information about the changes which occur in the CNS.

Given that, we performed our investigation using PBMC-derived monocytes, which represent a peripheral model of microglial cells' precursors. We explored the effect of D4T on modulation of Ras/MAPK and GSK3β/PI3K signaling pathways which downstream mediate Aβ autophagy using monocytes from AD patients and HC subjects following 2h LPS priming and 22h Aβ stimulation with or without D4T.

Results herein show that D4T can prevent the toxic effect due to Aβ exposure significantly reducing phosphorylation of p38 in both controls ($p<0.05$) (Fig.12A-B) and AD patients ($p<0.001$) (Fig.12A and C). In addition, D4T reduces p-p38 in patients cells compared to the related basal ($p<0.05$), while it decreased in HC ($p<.0.05$) (Fig.12B).

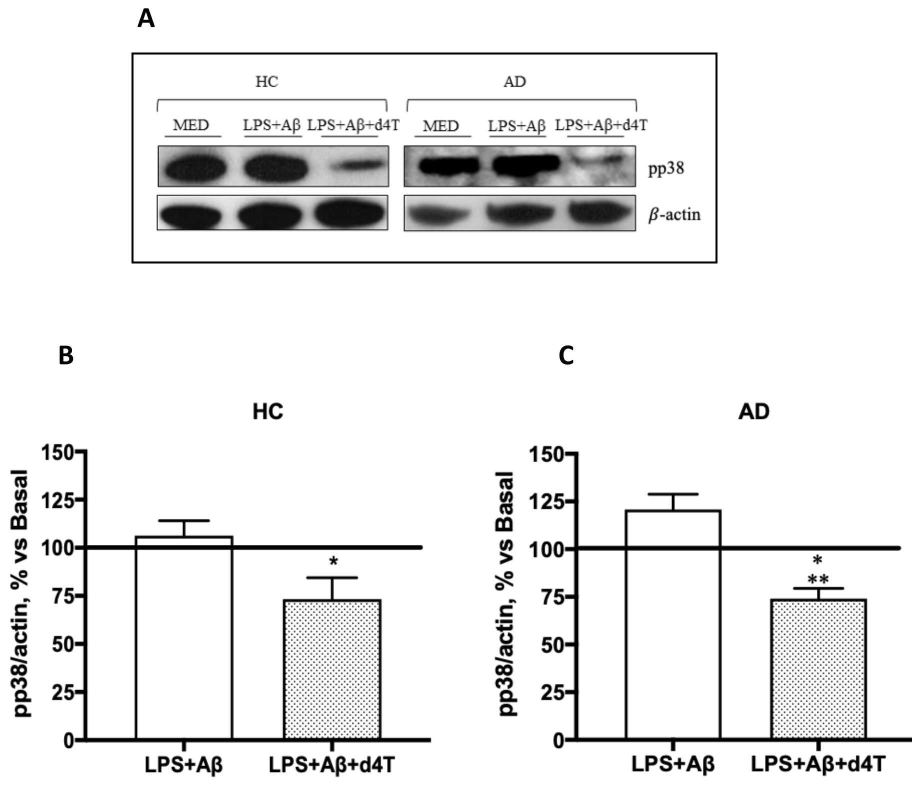
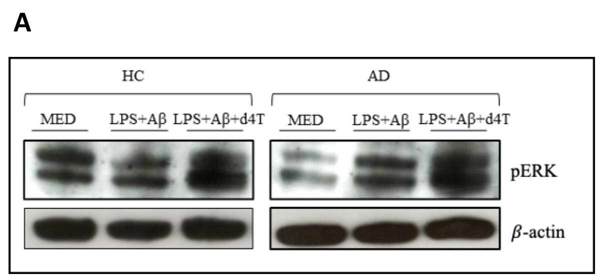


Figure 12A: Representative image of immunoblot of p38 phosphorylation state (38kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of p-p38/actin ratio vs basal in HC PBMC-derived monocytes, ANOVA with Tukey's post-test *p<0.05 vs LPS+Aβ; mean±SEM **C:** Data expressed as percentage of p-p38/actin ratio vs basal in AD PBMC-derived monocytes ANOVA with Tukey's post-test *p<0.05vs basal **p<0.001 vs LPS+Aβ; mean±SEM.

D4T up-regulated both p-ERK (p<0.001) (Fig. 13A-C) and p-AKT (p<0.02) (Fig. 14A-C) in HC and AD compared to LPS+Aβ stimulated cells. Moreover, D4T increased both ERK and AKT phosphorylation compared to the untreated basal in monocytes from AD patients (p<0.05) (Fig.14C).



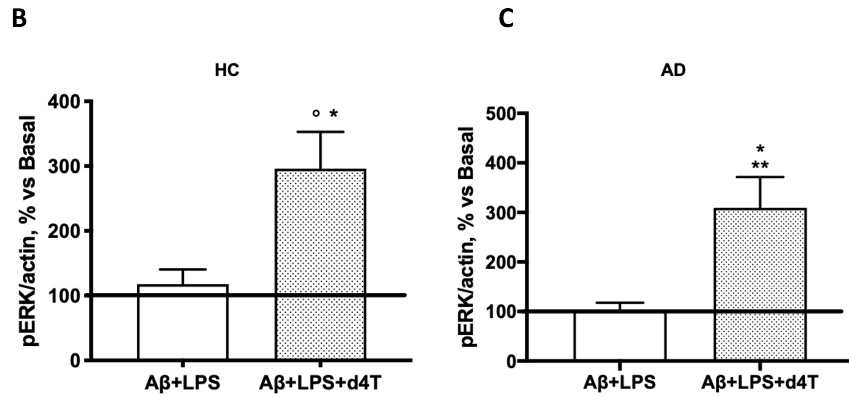


Figure 13A: Representative image of immunoblot of p-ERK phosphorylation state (42-44kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of p-ERK/actin ratio vs basal in HC PBMC-derived monocytes, ANOVA with Tukey's post-test °p<0.05vs basal *p<0.05 vs LPS+Aβ; mean±SEM **C:** Data expressed as percentage of p-ERK/actin ratio vs basal in AD PBMC-derived monocytes ANOVA with Tukey's post-test *p<0.05vs basal **p<0.001 vs LPS+Aβ; mean±SEM.

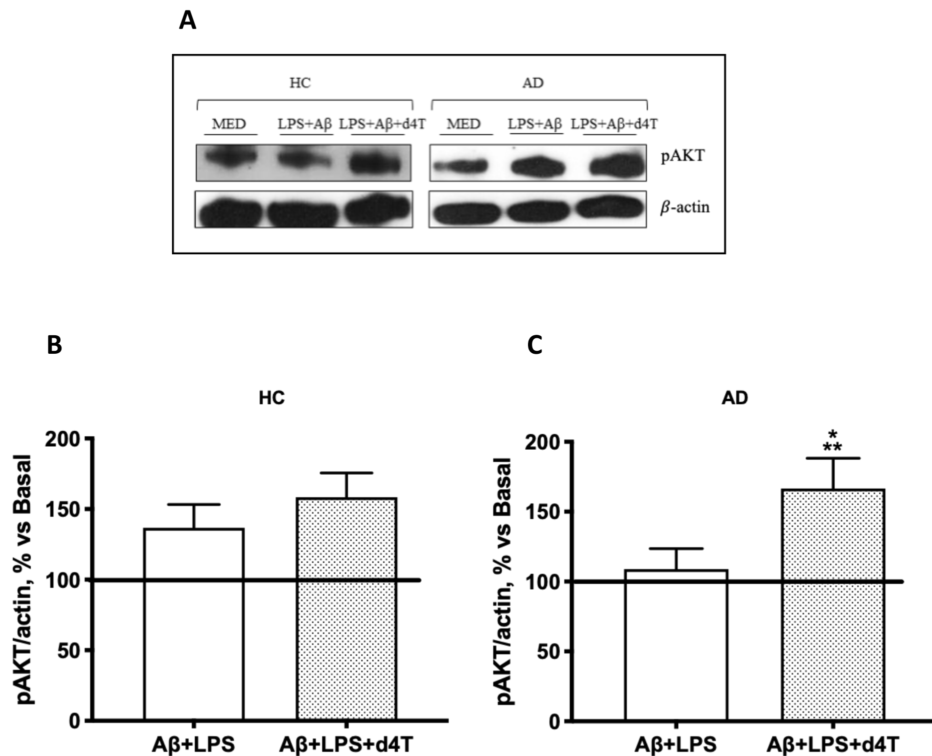


Figure 14A: Representative image of immunoblot of p-AKT phosphorylation state (60kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of p-ERK/actin ratio vs basal in HC PBMC-derived monocytes **C:** Data expressed as percentage of p-AKT/actin ratio vs basal in AD PBMC-derived monocytes ANOVA with Tukey's post-test *p<0.05vs basal **p<0.02 vs LPS+Aβ; mean±SEM.

- D4T impacts on autophagy signaling pathway

Beclin-1, LAMP2A and phospho-p70S6K signals, key autophagy regulators, were examined by western blot analysis in protein extracts from monocytes from AD patients and HC subjects following LPS priming and Aβ stimulation in the absence or presence of D4T.

Beclin-1 is a molecular platform which regulate the initiation of the autophagosome formation, the expression level and post-translational modifications of Beclin-1 regulate macroautophagy induction [364]. Results herein show that Beclin-1 was only marginally increased by D4T treatment in LPS-primed and Aβ-stimulated monocytes from AD patients (Fig. 15A and C), while any significant modulation due to D4T was detected in HC PBMC-derived monocytes.

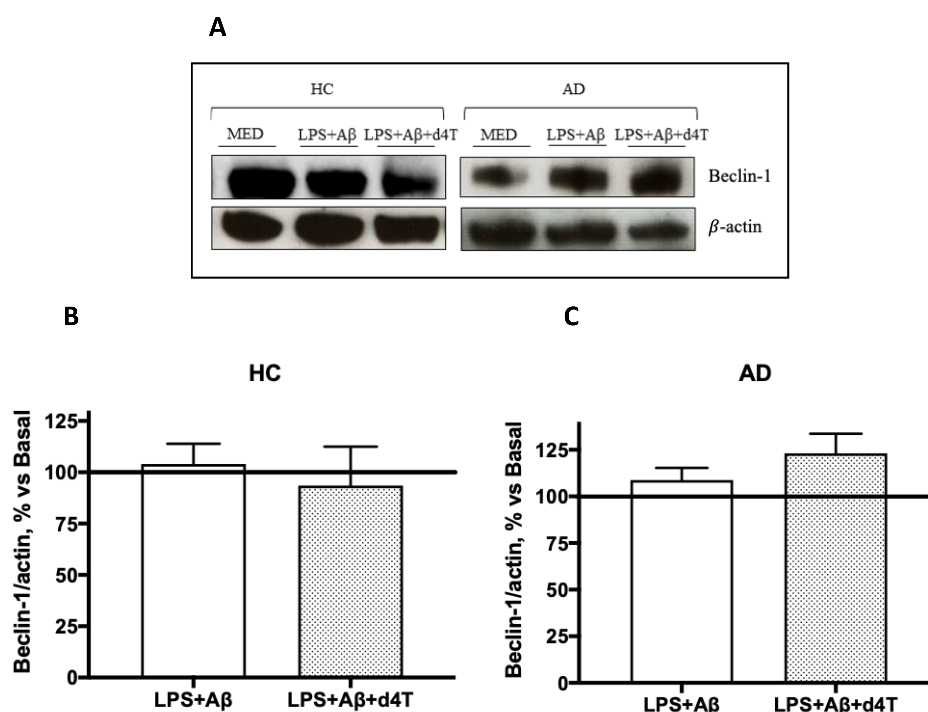


Figure 15A: Representative image of immunoblot of Beclin-1 expression levels (52kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of Beclin-1/actin ratio vs basal in HC PBMC-derived monocytes **C:** Data expressed as percentage of Beclin-1/actin ratio vs basal in AD PBMC-derived monocytes.

We also analyzed both p70S6-kinase and p85S6-kinase expression levels, which are ERK1/2 and AKT downstream effectors. In particular, the 70kDa isoform is involved in regulation of cell-cycle and also in the activation of Tau protein synthesis and its phosphorylation, while the 85KDa isoform exclusively nuclear and modulates the transcriptional mechanisms [320].

Our results shown that in HC monocytes and in AD as well, both phospho-p70S6K isoforms: the 70kDa cytosolic form and the 85kDa nuclear one, were significantly upregulated by D4T ($p<0.05$) (Fig. 16A, C and E) when they were compared to the respective unstimulated basal cells ($p<0.05$) (Fig. 16A, B and D).

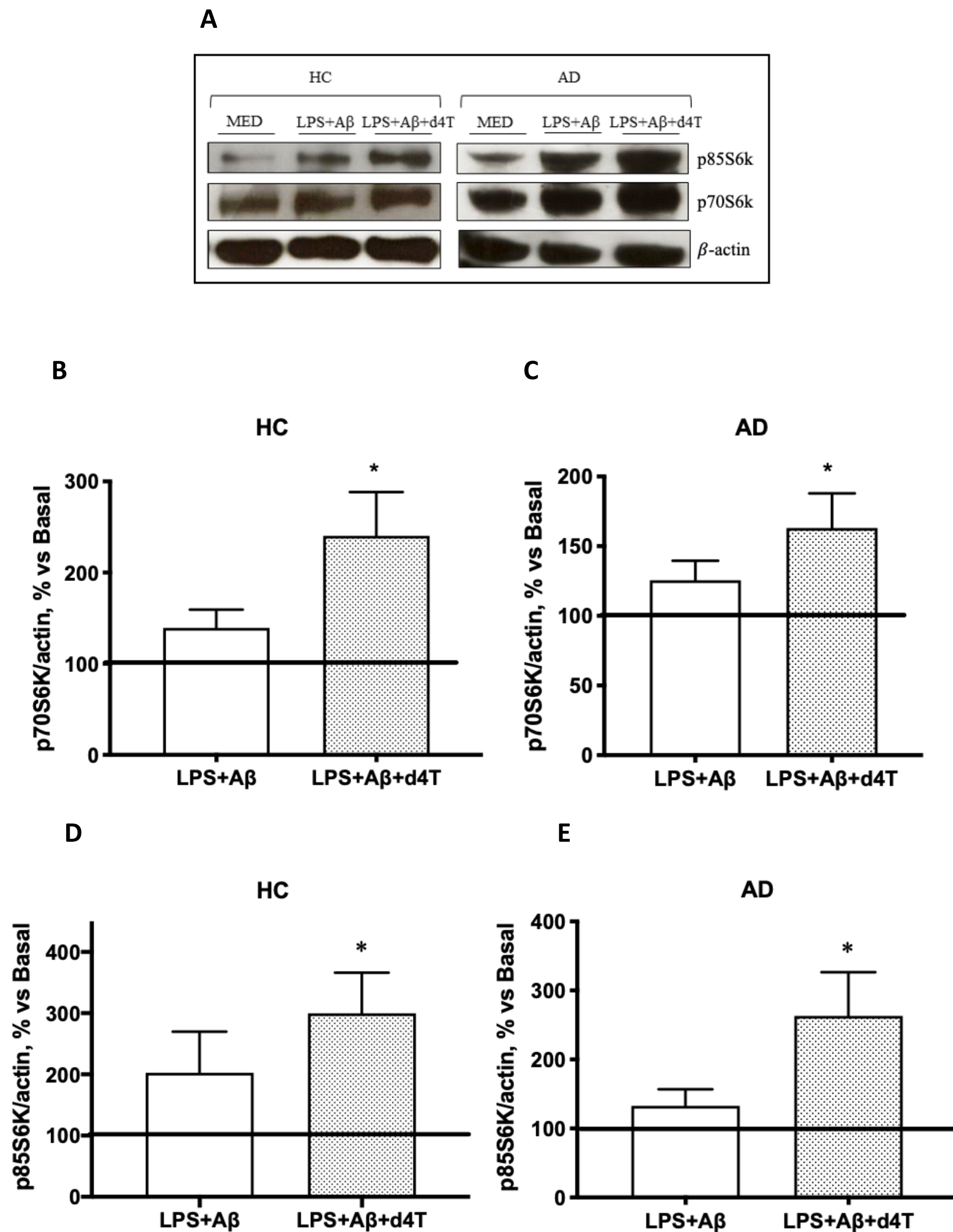


Figure 16A: Representative image of immunoblot of p70S6K and p85S6K phosphorylation state (70 and 85 kDa). Results were normalized to β-actin expression (42kDa). **B-D:** Data expressed as percentage of p70- and p85S6K/actin ratio vs basal in HC PBMC-derived monocytes ANOVA with Tukey's post-test *$p<0.05$ vs basal; mean±SEM. **C-E:** Data expressed as percentage of p70- and p85S6K/actin ratio vs basal in AD PBMC-derived monocytes ANOVA with Tukey's post-test *$p<0.05$ vs LPS+Aβ; mean±SEM.

Another target of our interest was LAMP2A. The expression of this channel protein can be also regulated by AKT signaling and plays a pivotal role in the chaperon mediated autophagy pathway (CMA), another

type of lysosomal degradation, in fact LAMP2A bind and translocate substrate proteins into the lysosome for degradation [522].

Our data shown that D4T increased LAMP2A expression in LPS primed and Aβ stimulated monocytes from HC ($p<0.001$) (Fig. 17A-B) and AD (Fig.17A-C) compared to monocytes exposed to cytotoxic stimuli ($p<0.001$) and to the respective unstimulated basal cells ($p<0.05$).

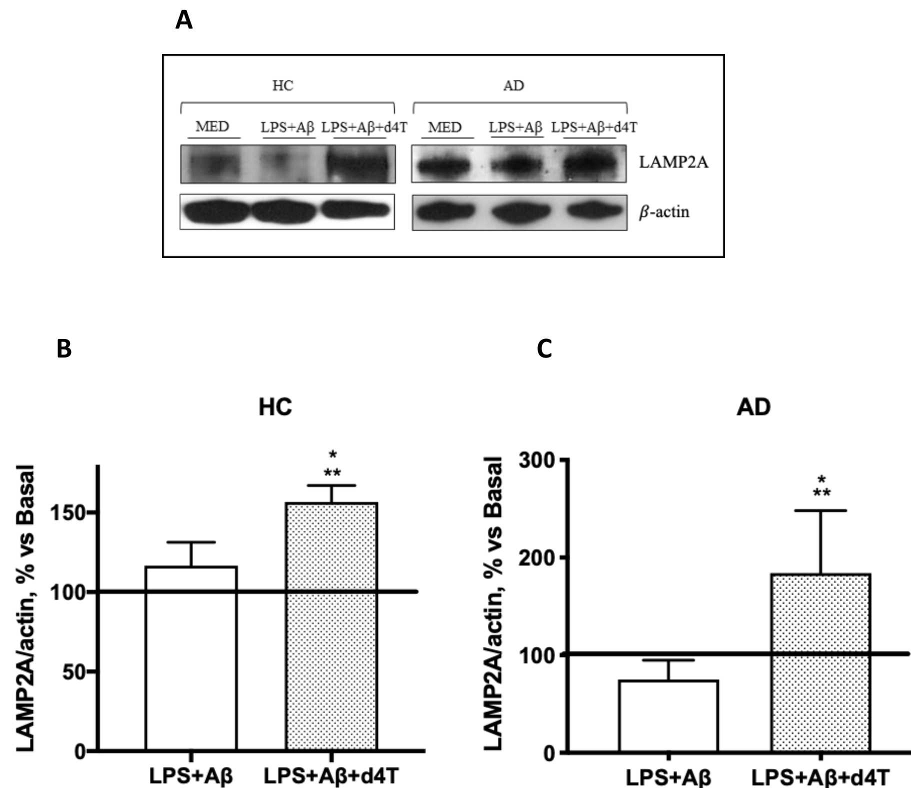


Figure 17A: Representative image of immunoblot of LAMP2A (100kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of LAMP2A/actin ratio vs basal in HC PBMC-derived monocytes, one-way ANOVA with Tukey's post-test *$p<0.05$ vs basal **$p<0.001$ vs LPS+Aβ mean±SEM. **C:** Data expressed as percentage of LAMP2A/actin ratio vs basal in AD PBMC-derived monocytes ANOVA with Tukey's post-test *$p<0.05$ vs basal **$p<0.001$ vs LPS+Aβ; mean±SEM.

- D4T effects on MEK and AKT downstream pathways

Protein expression of CREB and Bax, two ERK and AKT downstream mediators were also analyzed. CREB is a transcription factor which plays a pivotal role in memory acquisition and consolidation through the transcription of genes involved in synaptic plasticity and long-term memory [243].

Our data in shown that D4T induced pCREB in monocytes from HC and AD patients compared to the relative LPS primed and Aβ-stimulated cells ($p<0.01$) (Fig.18A-C). Regarding to the untreated basal, D4T increased pCREB in both HC and AD monocytes ($p<0.05$) (Fig. 18C).

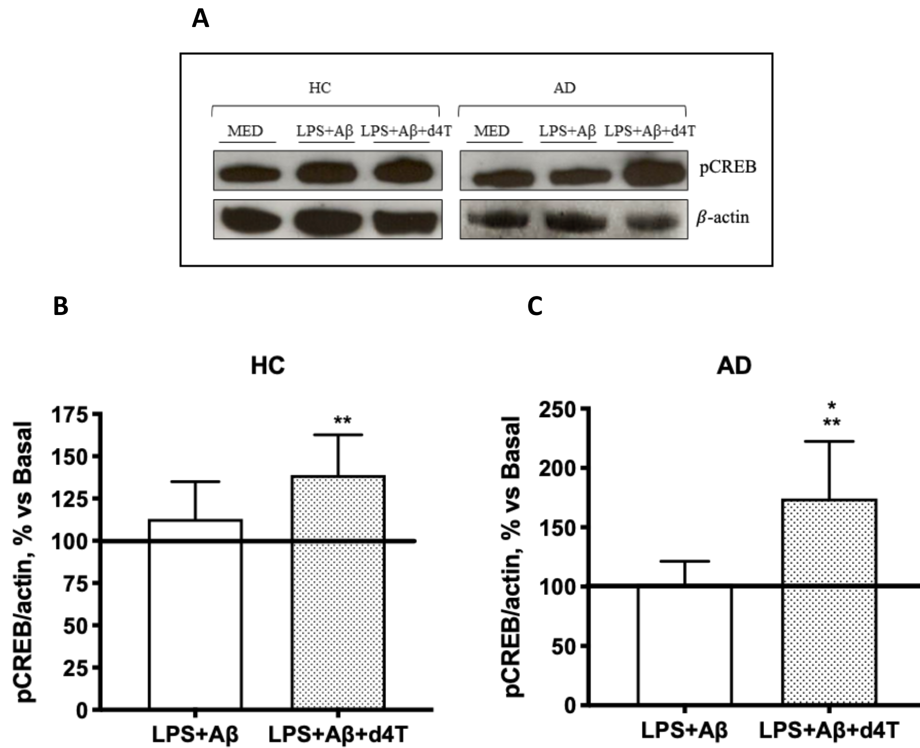
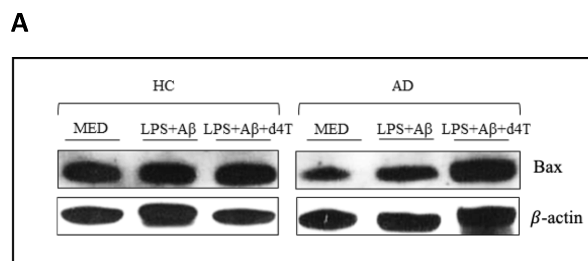


Figure 18A: Representative image of immunoblot of pCREB (43kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of pCREB/actin ratio vs basal in HC PBMC-derived monocytes, one-way ANOVA with Tukey's post-test $^{**}p<0.01$ vs LPS+Aβ mean±SEM. **C:** Data expressed as percentage of pCREB/actin ratio vs basal in AD PBMC-derived monocytes ANOVA with Tukey's post-test $^{*}p<0.05$ vs basal $^{**}p<0.01$ vs LPS+Aβ; mean±SEM

Bax belongs to the multi-BH domains pro-apoptotic subfamily, which promotes apoptosis by translocating into the mitochondrial membrane and facilitating cytochrome c release. It has been reported that reduction in Bax expression protects neurons from Ab-induced cell death [523].

In monocytes from AD patients, D4T increased Bax expression compared to the untreated basal ($p<0.05$) and the stimulated cells as well ($p<0.05$) (Fig.19A-C), suggesting that D4T could facilitate apoptosis to counteract Aβ cytotoxicity. No significant modulation has been detected in HC monocytes (Fig 19B).



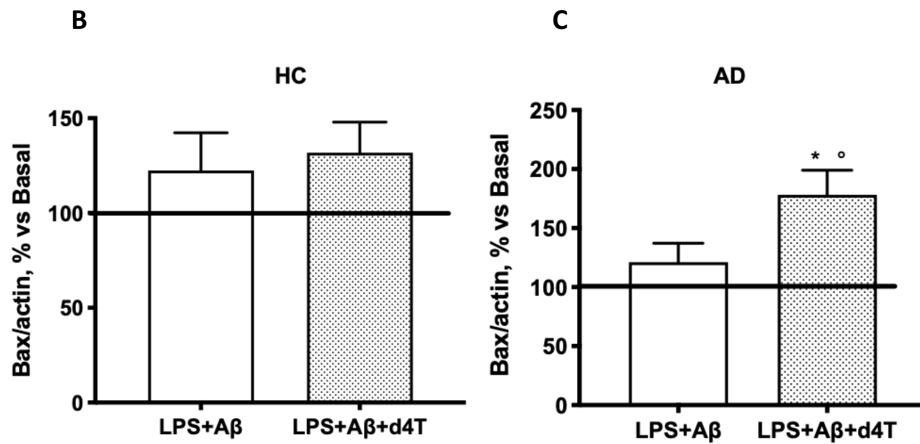


Figure 19A: Representative image of immunoblot of Bax (21kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of Bax/actin ratio vs basal in HC PBMC-derived monocytes mean±SEM. **C:** Data expressed as percentage of Bax/actin ratio vs basal in AD PBMC-derived monocytes, one-way ANOVA with Tukey's post-test °$p<0.05$ vs basal *$p<0.05$ vs LPS+Aβ; mean±SEM

- D4T modulation on TREM2

TREM2 is a cell surface receptor expressed by monocyte derived dendritic cells and microglia in the CNS, which is required for microglia migration and plays a pivotal role in cytokine release and phagocytosis [427]. Data herein show that, in our experimental conditions D4T had not any significant effect on TREM2 protein levels in both HC and AD monocytes (Fig. 20A-C).

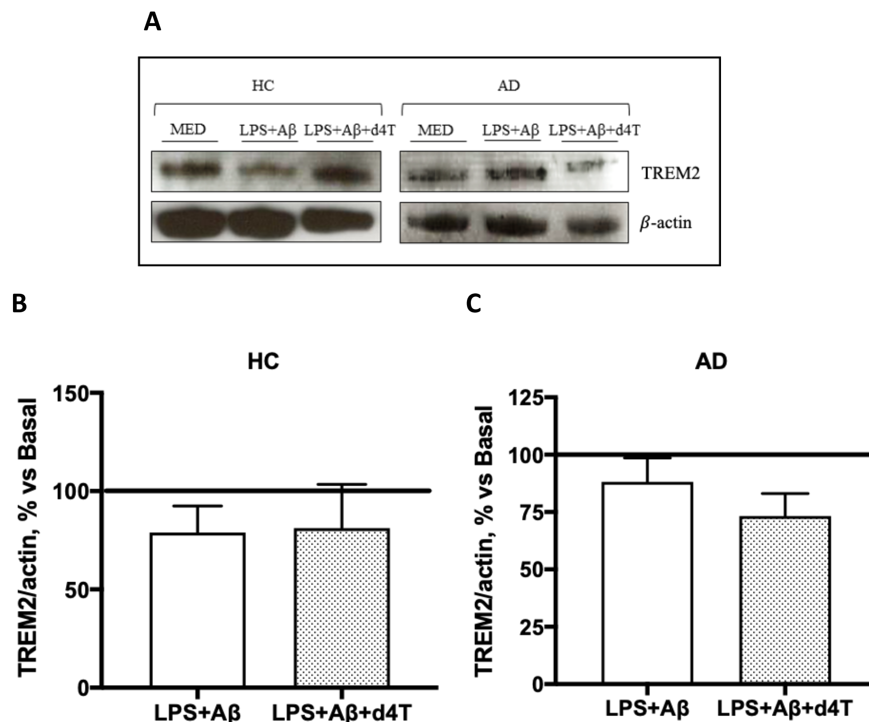


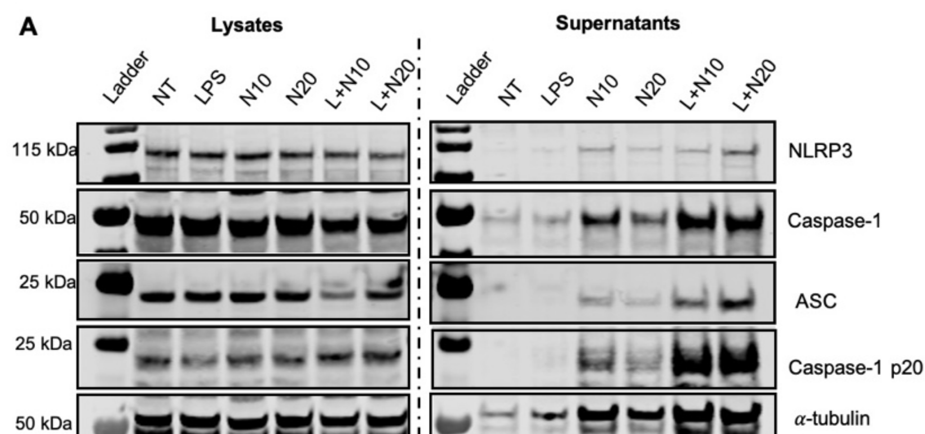
Figure 20A: Representative image of immunoblot of TREM2 (25-28kDa). Results were normalized to β-actin expression (42kDa). **B:** Data expressed as percentage of TREM2/actin ratio vs basal in HC PBMC mean±SEM. **C:** Data expressed as percentage of TREM2/actin ratio vs basal in AD PBMC, mean±SEM.

6.3 Investigation of NLRP3 inflammasome pathway activation in Aβ-related pathology

In the third part of this study, we focused our attention on the role NLRP3-inflammasome activation pathway by Aβ oligomer in AD pathogenesis. In particular, our goal was to characterize the NLRP3 inflammasome pathway activation and its interaction with other inflammatory pathways in AD using THP-1 cells and patient and healthy controls-derived monocytes-derived microglial cells (MDMIs).
Experiments were carried out in collaboration with Prof. Dr. Heneka's laboratory at the German Center for Neurodegenerative Diseases (DZNE) in Bonn, Germany.

- Inflammasome activation and phagocytosis tests in THP-1 cells

In order to characterize the NLRP3 inflammasome pathway activation in MDMIs, preliminary test experiments in THP-1 cells have been performed. In order to avoid NLRP3 inflammasome activation due to LPS only, we decreased LPS concentration and increased the incubation time, priming THP-1 cells for 3h with 100ng/ml LPS, thereafter cells were stimulated with increasing concentrations (10mM and 20mM) of Nigericin, a strong NLRP3 activator. By western blot, we observed a dose dependent increase of NLRP3, ASC, Caspase-1 ($p<0.05$) and the cleaved form p20 protein expression upon LPS priming and stimulation with Nigericin on total cell lysates and proteins precipitated from the RPMI-1640 supernatants, too (Fig. 21A-C). Moreover, we observed the same dose dependent trend on IL-1β release ($p<0.001$) on the supernatants suggesting a strong NLRP3 inflammasome activation (Fig 21D).



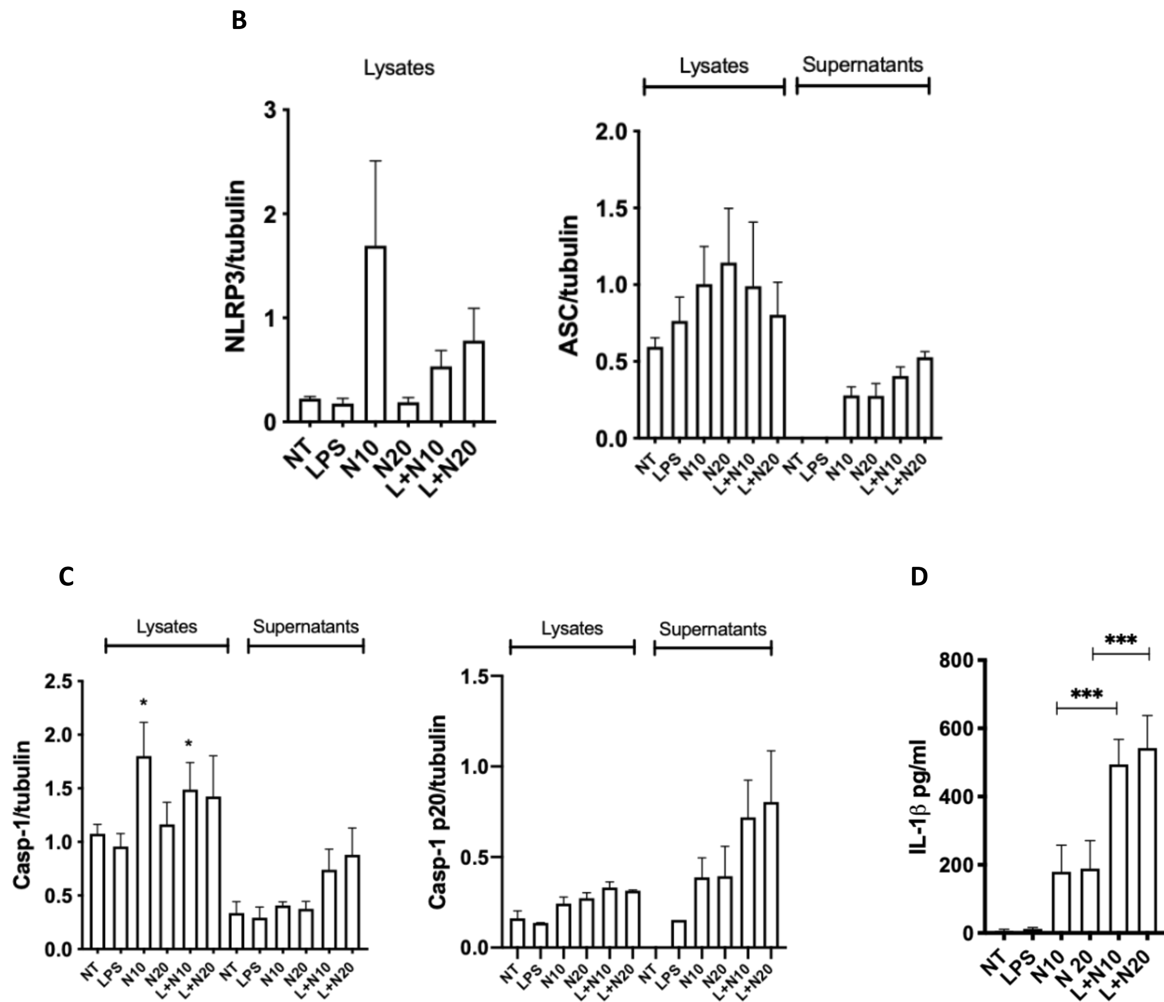


Fig 21 A-C: Representative image of immunoblot on cells lysates and supernatants, quantification of NLRP3, ASC (**B**) Caspase-1 and Caspase-1 p20 proteins expression (**C**). Signals were normalized to the α-tubulin expression (55 KDa) *p<0.05 vs NT. **D:** IL-1β release on cells supernatants; experiments carried out in triplicates; ***p<0.001 one-way ANOVA, mean ± SEM.

THP-1 cells can eliminate Aβ through phagocytosis. Thus, Aβ-FAM and Aβ-TAMRA phagocytosis were tested in order to identify the best fluorescent tagged peptide. Cells were pre-incubated for 30min with Cytochalasin D, a phagocytosis inhibitor, and exposed to Aβ-FAM, Aβ-TAMRA peptide and pHRodo beads for 6h, 4h, 2h and 30min. We observed an increase of Aβ-FAM phagocytosis in time dependent manner until 4h, after 6h degradation of the peptide may occur. While THP-1 cells did not seem engulf Aβ-TAMRA (Fig 22). Given that, Aβ-FAM was selected for further phagocytosis experiments in MDMIs.

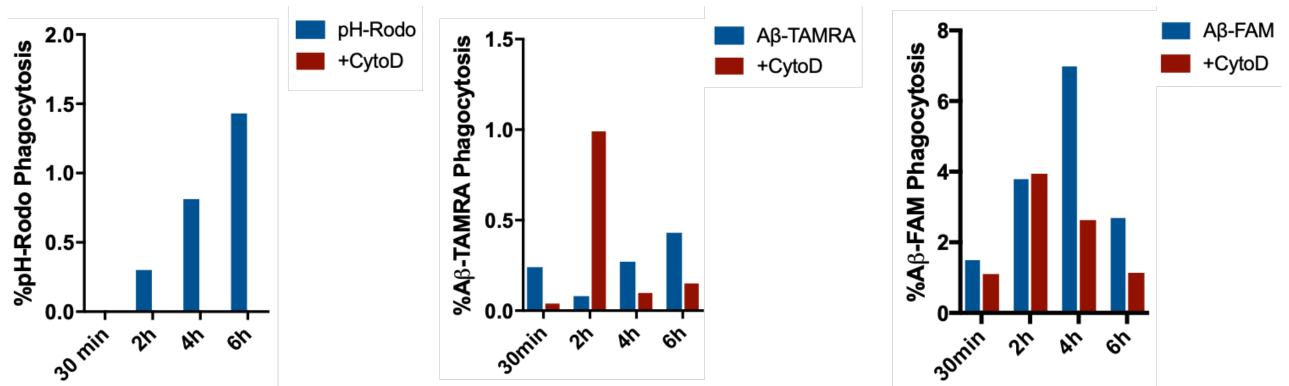
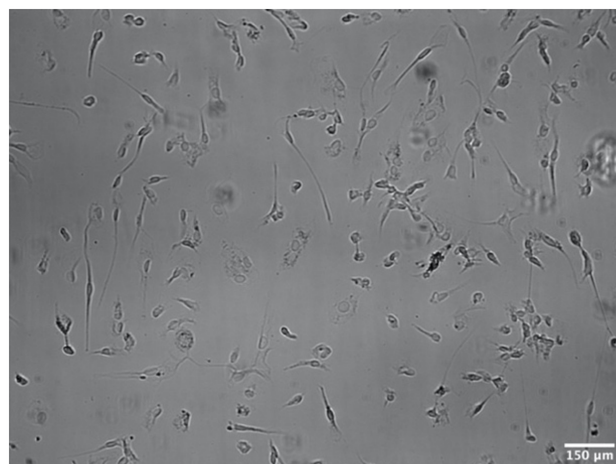


Fig 22: Percentage of engulfed Aβ-FAM and Aβ-TAMRA at different time points evaluated by flow cytometry, pHRodo beads were used as phagocytosis positive control. Data analysed by FlowJo software V.10.

- Monocytes-derived microglial cells express typical surface and intracellular myeloid markers

In order to characterize the expression of surface and intracellular microglial markers MDMIs were stained with a panel of different antibodies. By flow cytometry analysis (Fig.23B) and immunocytochemistry (Fig.23C-D) we found that these differentiated cells had a ramified morphology (Fig.23A) and displayed a myeloid phenotype, expressing typical myeloid surface markers (CD11b, CD45, CD14, CD16, HLA-DR); hematopoietic markers (CD43 and CD34), intracellular scavenger receptors (CD36 and CD68), and chemokine receptors (CCR2, CCR4, CX3CR1). Moreover, they also express the microglial markers TREM2, TMEM119, IBA1, Pu.1 and P2YR12.

A



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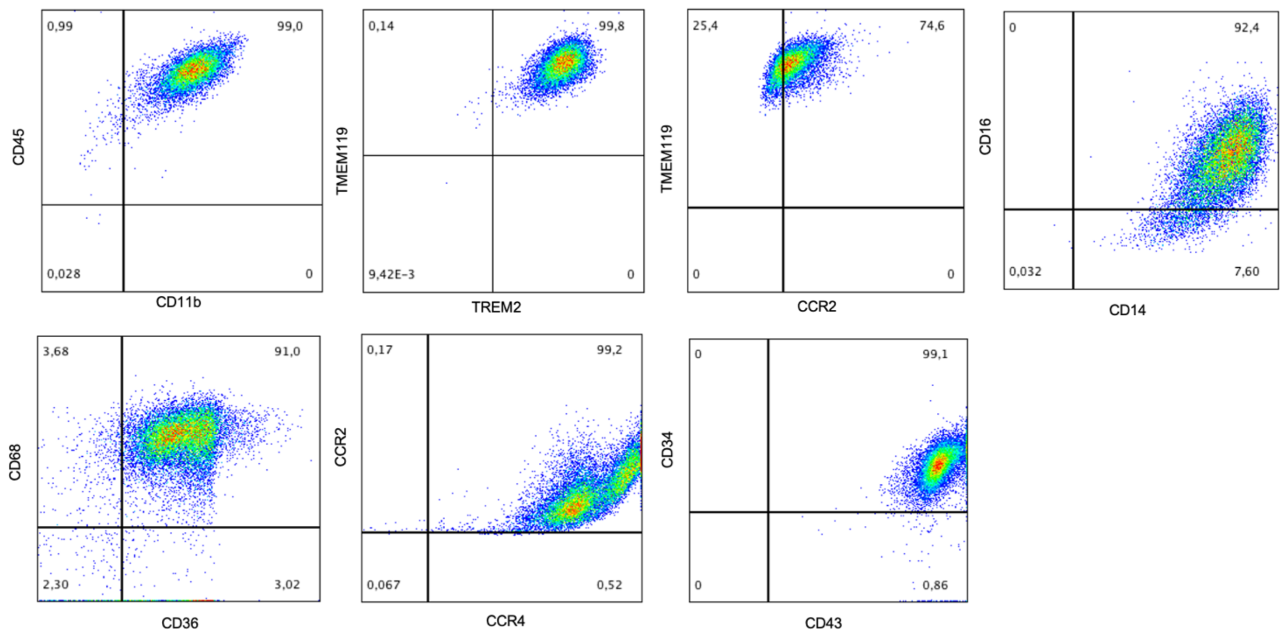

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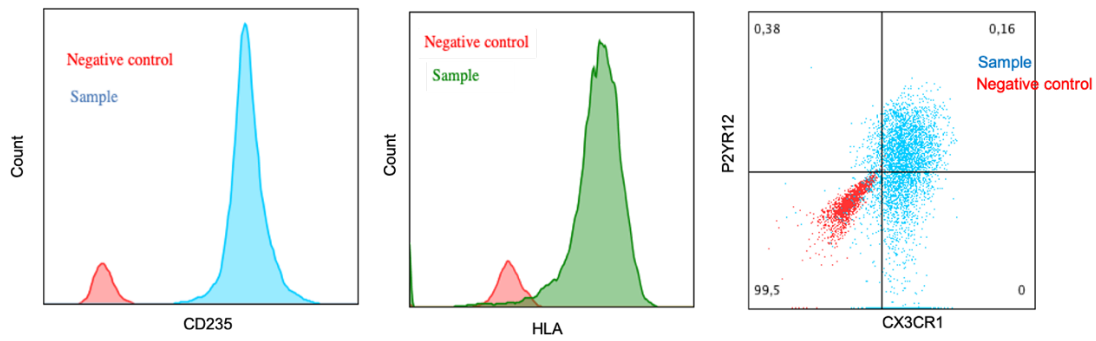

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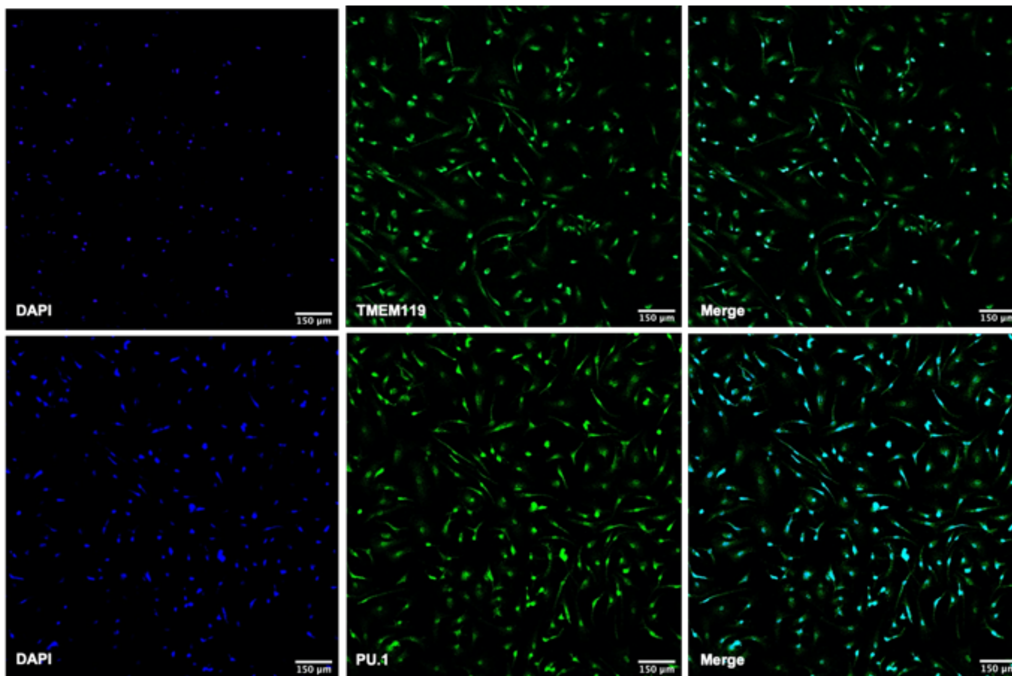

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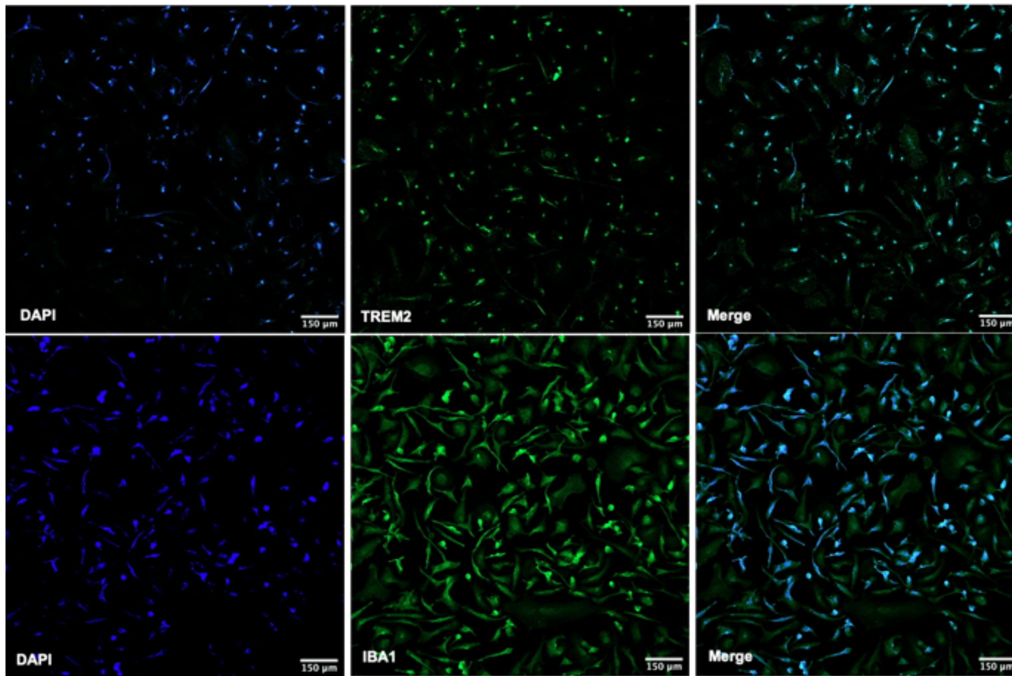


Figure 23A: Image representing monocytes-derived microglia cells (MDMIs) acquired using a Nikon Eclipse Ti fluorescence 20X microscope (Nikon). Scale bar: 150μm **B-C:** Representative images of flow cytometry dot plots and histograms representing myeloid markers expression in HC MDMIs. Data analyzed with FlowJo. **D-E:** immunocytochemical detection in AD MDMIs of TMEM119, Pu.1 in green (**D**), TREM2 and IBA1 in green (**E**), DAPI (blue) was used as a nuclear counterstain; scale bar: 150 μm.

- Analysis of the NLRP3 inflammasome pathway activation in MDMIs

In order to characterize NLRP3 inflammasome pathway activation, we investigated the expression of NLRP3-related proteins and the subsequent pro-inflammatory cytokines release. At this purpose, IL-1β and IL-18 pro-inflammatory cytokines release was measured on cell culture supernatants collected from HC and AD MDMIs. Unfortunately, the current pandemic situation has restricted patients and healthy subjects' recruitment, for this reason the expression of caspase-1; caspase-1 p20, its active form, and ASC proteins were detected in MDMIs cell lysates and supernatants obtained from AD patients only. We observed higher pro-inflammatory cytokines release in supernatants collected from AD MDMIs compared to HC ($p<0.001$) upon LPS priming and ATP and Aβ stimulation (Fig 24A). Proinflammatory cytokines IL-1β and IL-18 production was increased both in Aβ and ATP stimulated MDMIs from AD patients and HC following LPS priming compared to untreated cells ($p<0.001$) (Fig. 24B-C), while CRID3 treatment could counteract pro-inflammatory cytokines release in HC and also AD MDMIs ($p<0.001$) (Fig. 24A-C).

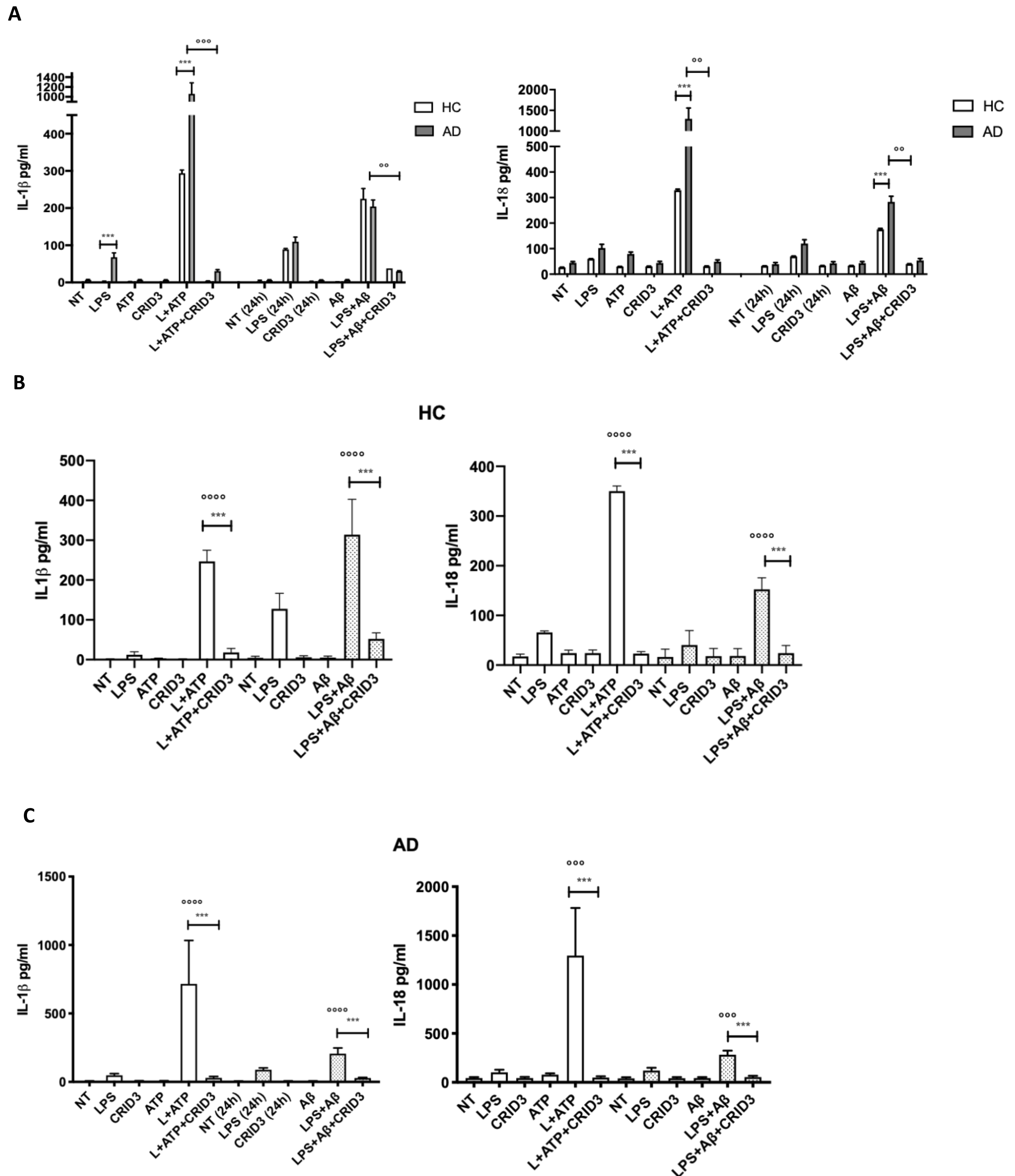
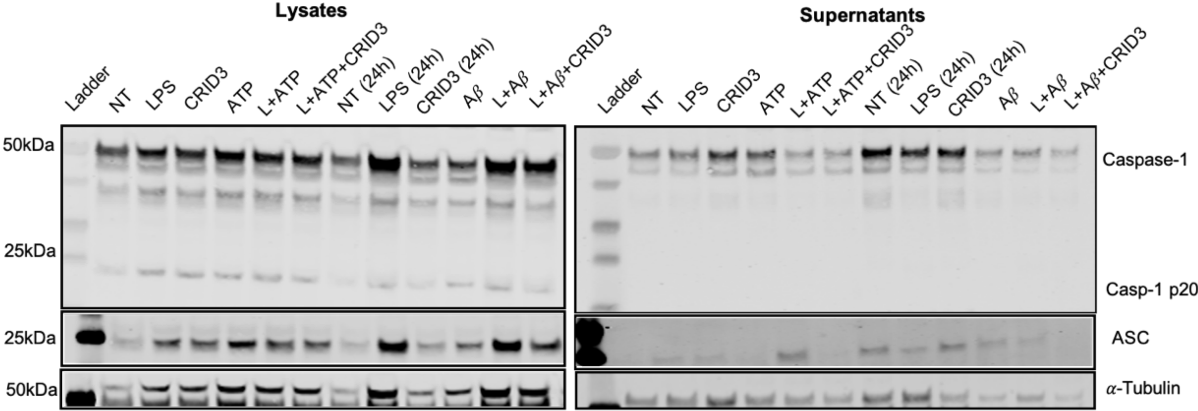


Fig 24A: IL-18 and IL-1β release on HC vs AD MDMIs supernatants; experiments carried out in triplicates; ***p<0.001 AD vs HC; °°p<0.01 L+ATP vs L+ATP+CRID3 °°°p<0.001 L+Aβ vs L+Aβ+CRID3 **B:** IL-1β and IL-18 release on HC MDMIs supernatants. Experiments carried out in triplicates; ***p<0.001; °°°°p<0.0001 vs NT. **C:** IL-1β and IL-18 release on AD MDMIs supernatants. ***p<0.001 L+Aβ vs L+Aβ+CRID3; ***p<0.001 L+ATP vs L+ATP+CRID3; °°°p<0.001 vs NT; °°°°p<0.0001 vs NT. Two-way ANOVA, mean ± SEM.

NLRP3-related proteins expression was also investigated upon ATP and Aβ stimulation following LPS priming in AD MDMIs (Fig 25A-C). Results showed an increasing trend in caspase-1, its active form

caspase-1 p20 and ASC following LPS priming and ATP and Aβ stimulation (Fig 25B). While CRID3 seems to reduce ASC protein expression due to ATP and Aβ stimulation in MDMIs from AD patients (Fig 25C). Unfortunately, due to the low sample size it is not possible to obtain statistical significance. The data herein shown only reveals a trend of the effect of CRID3.

A



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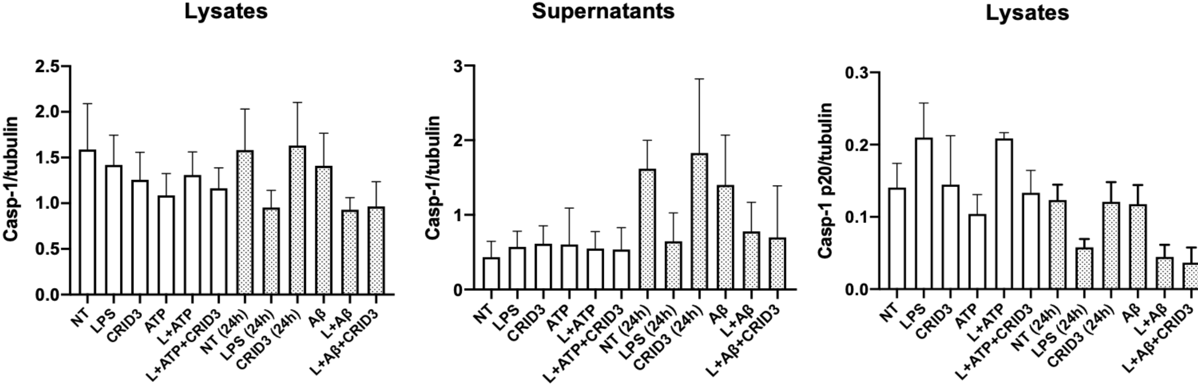

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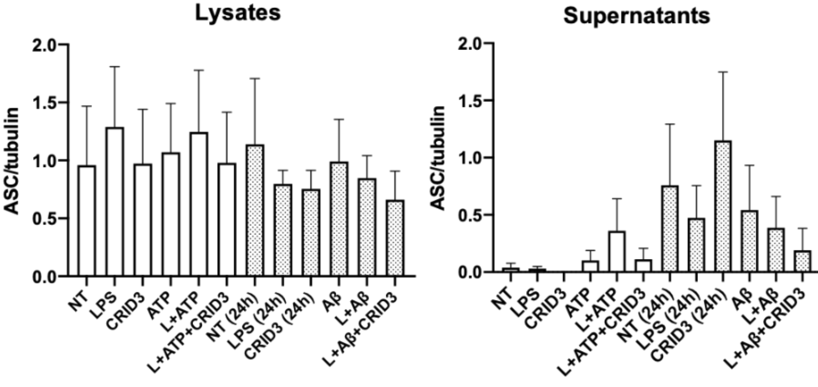
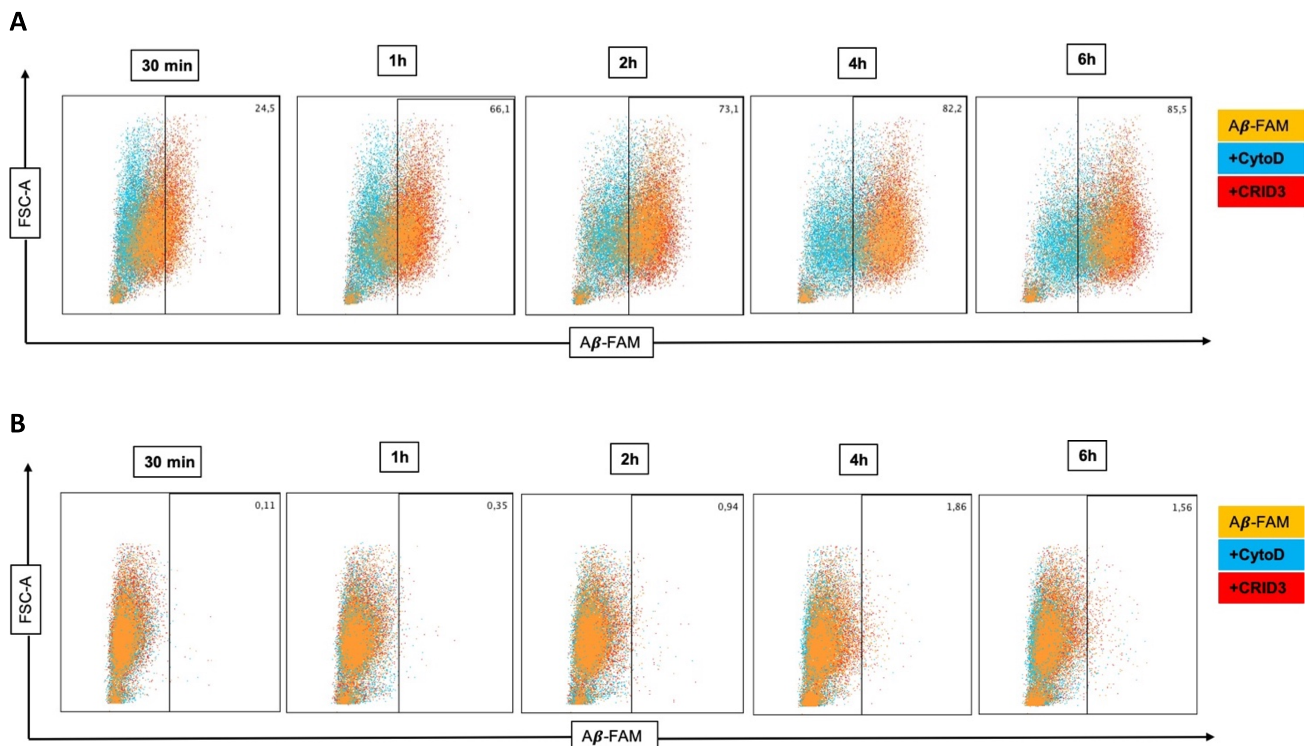


Fig 25A-C: Representative images of immunoblots of Caspase-1 (45kDa), ASC (20-25kDa) and Caspase-1 p20 (20kDa) on AD MDMIs cells lysates and supernatants. **B-C:** quantification of Caspase-1, Caspase-1 p20 and ASC protein levels. Signals were normalized to the α-tubulin expression (55 KDa); mean ± SEM.

- Aβ Phagocytosis and degradation in MDMIs from AD patients

MDMIs can ingulf Aβ peptide through phagocytosis and degradation, but NLRP3 activation negatively affects the microglial clearance function [524]. We analyzed the modulation of this process inhibiting inflammasome activation, treating MDMIs obtained from AD patients with CRID3, a selective NLRP3 inhibitor. Cells were pre-incubated for 30 min with Cytochalasin D which inhibits phagocytosis and exposed to Aβ-FAM peptide for 6h, 4h, 2h, 1h and 30 minutes, with or without CRID3. MDMIs shown increased Aβ uptake in time-dependent manner as well as in CRID3 treated cells, while Cytochalasin D pretreatment dampened Aβ uptake ($p<0.001$) (Fig. 26A and C). Any significant impact of CRID3 treatment was detected on Aβ phagocytosis in our experimental condition. In addition to phagocytic uptake, we determined the degradation capacity of MDMIs, assessing the degradation of the internalized Aβ 17h after the peptide exposure. Results shown that only a small fraction of Aβ-FAM was still inside the cells upon longer time of Aβ exposure (4h and 6h) (Fig.26B and D), suggesting that MDMIs are able to clear Aβ and the clearance rate depends on the amount of the engulfed peptide.



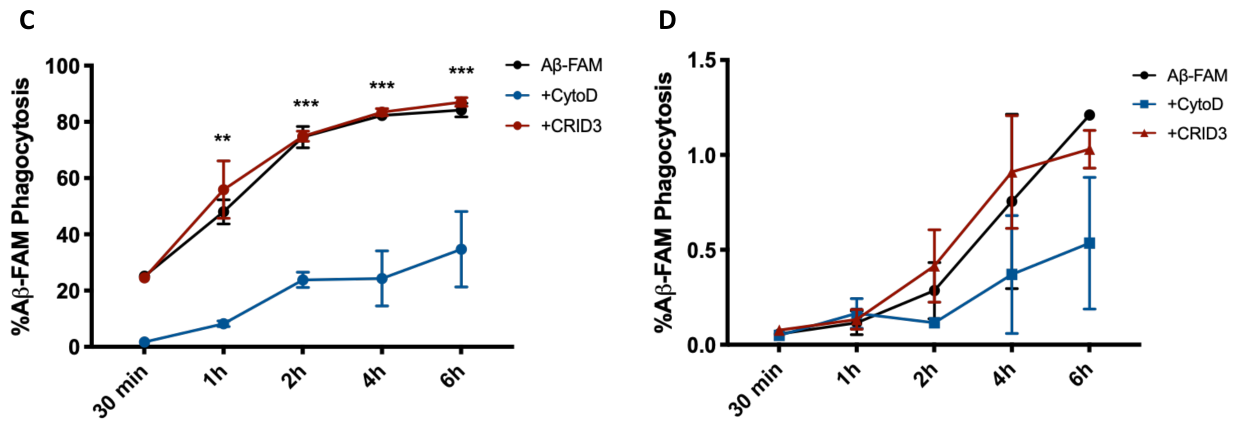


Figure 26A: Representative dot plots demonstrating the engulfment of Aβ-FAM by AD MDMIs. **B:** Representative dot plots demonstrating the engulfment of Aβ-FAM by AD MDMIs after 17h degradation. **C-D:** Quantification of MDMIs engulfing Aβ overtime (**C**) t-student test **p<0.01 ***p<0.001 vs CytoD, and after 17h of degradation (**D**).

Chapter 7
Discussion

7.1 Main study

The overall aim of our study was to characterize the complex interactions between Aβ oligomers and intracellular signaling in sporadic AD patients, with different disease severity, in order to evaluate any molecular pathways and related drugs, that could prevent or modulate Aβ-induced toxicity. We have investigated different molecular mechanisms using a combination of in vitro and specific ex vivo cellular models from sporadic patients and HC subjects, such as human primary fibroblasts, peripheral cells that in adequate conditions, express some of the molecular and biochemical alterations, observed in the CNS of AD patients [500,525]. Fibroblasts represent an outstanding opportunities for longitudinal mechanistic studies, since they could be kept in culture for a long time and could be differentiated into patient-specific neural cell lines using inducible pluripotent stem cell (iPSC) technologies [526,527].

The existing bi-directional communication between the brain and the periphery provides the chance to investigate central alterations at systemic level. Since monocytes provide a peripheral model of central myeloid cells precursor of microglia [528], we isolated PBMC-derived monocytes from AD patients and HC subjects to perform our analysis. Furthermore, we differentiated monocytes into microglia-like cells which represent a versatile and attractive in vitro model of human adult microglia and enable the study of microglia in vitro [529]. The human neuronal cell line (SH-SY5Y) and human macrophage-derived THP1 cells have been also employed for preliminary studies and to set up the proper experimental methods.

Because Aβ accumulation induces the activation of NLRP3 inflammasome complex in microglia, which is a key contributor to the development of neuroinflammation, therefore particular interest was given also to the analysis of NLRP3-inflammasome activation and modulation pathways. At this purpose, we investigated the possible involvement of NLRP3 inflammasome activation on Ras/MAPK and PI3K/AKT signaling and their downstream pathways such as autophagy, apoptosis, cytokine release, intracellular homeostasis and protein epigenetic modulations; and the related effects of two different NLRP3 inhibitors: the nucleoside reverse transcriptase inhibitor Stavudine (D4T) and CRID3. D4T reduces NLRP3 inflammasome activation, blocking P2X7R [488] and preventing the transcription caspase-1 and the subsequent maturation of IL-18; whereas the selective NLRP3 inhibitor CRID3, interacts directly with the NACHT domain of NLRP3, thereby blocking ATP hydrolysis [486]. Furthermore, CRID3 is highly specific for NLRP3 since it does not inhibit any other identified inflammasomes [487].

7.2 Intracellular signal transduction and potential protective role of Hop extract against Aβ cytotoxicity

A wide range of evidences has demonstrated that compromised Ras/MAPK and PI3K/AKT signaling pathways play key roles in the pathogenesis of various diseases including neurodegenerative disorders such as AD [219,494,530].

It has been reported that, PI3K/AKT is linked to tau hyperphosphorylation [531], whereas Ras/MAPK are triggered by $A\beta_{42}$ and contribute as cascade to neuroinflammation [264]. ERK is involved in APP metabolism through the regulation of α-secretase activity and it also modulates the glutamate transporter EAAT1 expression by phosphorylating CREB.

First of all, we investigated a panel of proteins in the MAPK and PI3K/AKT cascades, in fibroblasts from AD patients and MCI subjects compared to HC, highlighting the role of these pathways in Aβ-induced oxidative stress, APP metabolism, Tau phosphorylation and their involvement in the etiopathogenesis and progression of the disease. In particular, since the early stages of AD, p38 and JNK SAPK increased their phosphorylation state and a similar trend has been shown in MCI cells, these modifications may be due to cell bio-energetic impairment but any correlation to the disease severity has been detected. Several research teams, using human post-mortem tissues from AD patients, confirmed the observations that in human AD brains the p38 MAPK activation occurs from the early stage of the disease [265,266]. Furthermore, it has been reported that p38 MAPK signal transduction plays a pivotal role in Aβ-induced neuroinflammation in microglial cells [272]. In fact phosphorylated JNK and p38 in activated microglia, observed from the early stage of the disease, induce the production of pro-inflammatory factors which are toxic to neurons [532].

Several lines of evidence support a role for the ERK signaling pathway activation in the pathogenesis of AD. Aβ can induce an overactivation of the RAS/ERK signaling cascade [235] and high levels of activated ERK1/2 have been found in neurons and dystrophic neuritis from AD patients [238]. Furthermore, high levels of phosphorylated ERK (p-ERK) have been also found in degenerating neurons, in APP mouse models [235], in CNS from patients [236]. In line with these data, we analyzed the specific ERK1/2 involvement in AD fibroblasts. We observed that ERK1/2 phosphorylation is linked to the disease severity: p-ERK was increased in fibroblasts from severe AD patients compared to mild, moderate and healthy controls ones, whereas it was reduced in MCI, mild and moderate AD fibroblasts. Noteworthy, we observed an inverse correlation between MMSE score and p-ERK levels in AD patients and converter MCI, instead in non-converter MCI this correlation was lost, which strengthened the modulation of ERK1/2 signaling in different disease stages.

ERK1/2 is involved in APP metabolism, the regulation of EAAT1 glutamate transporter expression, the inhibition of inflammation and protection against oxidative stress [533], and its enhanced phosphorylation in patients with more advanced AD have been reported in neuronal cell bodies and dystrophic neurites around Aβ plaque [534]. In fact, an imbalance between the kinases and the phosphatase system has been reported in the fibroblasts of patients with severe AD [535], then we assume that a compensatory mechanism may occur as a subsequent attempt of the cells to balance the oxidative stress induced by Aβ deposition, which is involved in the inflammatory mechanisms in AD pathogenesis [236].

PI3K/AKT pathway plays a pivotal role in mediating neuronal survival and upstream regulates the mTOR-p70S6K signaling pathway, modulating autophagy and Tau protein phosphorylation [287] in neurons and in glial cells [237].

In agreement with these data, our results have shown the upregulation of PI3K/AKT phosphorylation (p-AKT) signaling pathway in fibroblasts from severe AD patients compared to HC cells while, in the early stages of the disease, an increasing trend has also observed. On the contrary, p-AKT status was reduced in MCI. Moreover, a positive correlation between p-AKT and the disease severity was also observed.

Hyperactivation of the PI3K/AKT increases also Bax expression, regulating apoptosis [327]. Abnormal activation of PI3K/AKT/mTOR pathway is linked to synaptic loss and cognitive decline in AD brains [299] and p-AKT is increased in hippocampal and cortical neurons in AD brains [300], and it is associated to enhanced Aβ deposition, due to the autophagy impairment [301]. The phosphorylation of p70S6K at T389 is a hallmark of activation by mTOR and correlated with autophagy inhibition [536]. Moreover, the distribution of activated p70S6K in human brain is linked to neurofibrillary degeneration, since abnormal p70S6K phosphorylation at T389 has been also associated with hyperphosphorylated tau in AD patients [323].

Our results have shown an increasing of p-p70S6K in fibroblasts from AD patients compared to HC. These data could be correlated to the increase of p-AKT previously mentioned. Moreover, we observed the induction in the cytoplasmic levels of Bax only in fibroblasts from AD patients, suggesting the triggering of apoptosis [518]. These results could be due to the activation of p38 and JNK pathways that integrate with the modulation of ERK1/2 and AKT signal transduction.

As a matter of fact, ERK1/2 is linked to APP metabolism and promotes the non-amyloidogenic APP processing by α-secretase [241]. Our results have shown a significant reduction of the αAPP levels, in particular in fibroblasts obtained from severe patients, compared to healthy subjects. p-ERK and αAPP levels correlated in HC subjects, demonstrating the ERK1/2 involvement in αAPP metabolism, whereas in MCI subjects and AD patients this correlation was lost. This might be related to the disease progression that impairs α-secretase activity. As previously mentioned, we observed that in MCI subjects that converted to AD, p-ERK levels correlated with MMSE score, while it did not show in non-converter MCI. We propose that this parameter might be helpful to predict the progression of MCI status, suggesting p-ERK as a useful peripheral biomarker.

The assessment of p-AKT and p-ERK levels in fibroblasts from AD patients may be a molecular support to clinical investigations and it might be also useful to better understand their relationship with Aβ toxicity and Tau hyperphosphorylation [537].

Molecular compounds able to regulate the abnormal activation of these pathways might be useful therapeutic strategies in the treatment of this neurodegenerative disorder.

Because of the lack of a disease-modifying therapy for dementia, preventive approaches such as diet, physical exercise, and learning are being explored. In the last decade, several studies focused their attention on the potential role of natural compounds to suppress neuroinflammation and improve cognitive functions [206,538].

Emerging evidences suggest that flavonoids, a group of phytochemicals compounds, are able to induce improvements in memory acquisition, consolidation and storage [503]. Moreover, they are effective in reversing age-related declines in memory, activating intracellular signaling pathways which regulate synaptic plasticity [539]. Hop (Humulus Lupulus) which is used as both a natural preservative and a flavoring agent in the beer-brewing process, contains flavonoids, aromatic molecules which have antioxidant, antinflammatory and antiatherogenic properties [204]. Furthermore, it also modulates specific intracellular signaling pathways, such as ERK1/2-CREB pathway and the PI3K/mTOR cascade, underlying neuronal survival and cognitive performances [197,208]. In addiction it has been reported that hop extracts may interact with Aβ oligomers preventing their aggregation and production in "in vitro" cell lines [502].

Given that, we assessed the antioxidant and anti-inflammatory properties of hop extracts and their involvement in intracellular signaling pathways, which are deregulated in AD, in order to evaluate any molecular mechanisms that could prevent or modulate Aβ-induced toxicity.

Human SH-SY5Y neuroblastoma cells have been employed as a CNS cellular model to investigate the role of Hop extract (HT) on energetic metabolism and on the activation of PI3K/AKT and ERK1/2 signaling pathways as well as p70S6K, their downstream target.

Short-term treatments with HT reduced both ERK and AKT phosphorylation. The down-modulation of these signaling pathways results in the reduction of mTOR inhibition thereby inducing macroautophagy. Regarding p-p70S6K, we observed that it is also reduced upon short-time exposure to HT, and p70S6K phosphorylation levels are still decreased after 24h, as observed for p-ERK. Given that, we can assume that Hop modulates key intracellular signaling pathways which are deregulated in AD. However, further investigations are required to test whether this compound may have a protective role in mediating autophagy and reducing tau hyperphosphorylation.

Thereafter, we tested the potential protective role of hop extracts against Aβ cytotoxicity on fibroblasts obtained from HC, MCI and AD patients. Previous data obtained in our laboratory have been demonstrated that 5 μM Aβ oligomers are able to induce biomolecular modifications in HC fibroblasts as observed in fibroblasts from AD patients [516], reducing cell viability and energetic metabolism. In line with these data, we observed that acute exposure to Aβ oligomers significantly reduces the functionality of energetic metabolism in fibroblasts, while HT treatment seems to prevent this toxic effect, restoring the proper mitochondrial functions at the basal level. We have hypothesized that the protective effect of hop extracts on Aβ toxicity is due to the antioxidant action of the flavonoids content in hops which improve mitochondrial activity, interacting with amyloid protein aggregates or binding to Aβ receptors, as an

agonist drug, counteracting Aβ induced oxidative stress [540], the subsequent toxic action on cellular metabolism and the glutamate transporter EAAT1 impairment, that is energy dependent.
Therefore, we exposed fibroblasts from recruited AD and MCI subjects to HT for 24h in order to investigate HT involvement in PI3K/AKT and MAPK signaling pathways. Our data shown reduced phosphorylated p38 SAPK upon HT treatment in AD fibroblasts, which is activated since the early stage of the disease, confirming that hop extracts might reduce oxidative stress related pathways.
Previous results, obtained in fibroblasts from mild/moderate AD patients, revealed a decreasing in p-ERK, following hop exposure it increased. As we just said, ERK1/2 is involved in regulation of α-secretase activity and glutamate transporter EAAT1 expression, by phosphorylating CREB which regulates EAAT1 transcription and expression. Thus, p-ERK increase could enhance the glutamate uptake, also at the peripheral level. In fact, glutamate is also the substrate required for the production of glutathione, which is essential to counteract oxidative stress [500]. We suppose that hop treatment might be beneficial in AD preclinical stage, promoting the non-amyloidogenic pathway and preventing Aβ accumulation. In addition, the anti-inflammatory action of ERK could be helpful in the later stages of the disease, reducing the pro-inflammatory cascade triggered by Aβ.
Regarding PI3K/AKT signaling pathway, we observed that AKT is highly phosphorylated in AD fibroblasts. The overactivation of AKT in AD brains suggests that neurons might activate this prosurvival pathway to counteract Aβ induced damages [541]. However, this excessive and non-selective phosphorylation of AKT could be deleterious to neuronal survival, leading to the subsequent deregulation of AKT downstream targets, among them GSK3, mTOR and Bax, which regulate cell survival, autophagy and apoptosis [300]. In addiction abnormal p-AKT levels in AD patients are associated with the tau hyperphosphorylation and the subsequent development of neurofibrillary pathology [542].
Our results in fibroblasts exposed to HT treatment have shown that hop extracts were able to reduce AKT phosphorylation status in MCI fibroblasts only, while any significant modulation has been detected in AD cells. We assume that hop treatment cannot counteract alterations in PI3K/AKT signaling pathway at the progressive stages of the disease, while it might be useful in AD preclinical stage.

7.3 Involvement of NLRP3 inflammasome activation on Ras/MAPK and PI3K/AKT pathways and autophagy modulation

Beyond investigating the detrimental impact of Aβ oligomers in the intracellular signaling pathways that are also involved in the anti-inflammatory mechanisms, in the second part of this study we have directed our attention on mechanisms involved in neuroinflammation.
Several studies have been focused on mutual interaction of innate immune mechanisms and neurodegenerative processes, demonstrating that activated microglia and the subsequent innate immune

response play a pivotal AD pathogenesis and progression [462]. Microglia can sense Aβ expressing receptors that promote Aβ clearance and phagocytosis, although during AD pathogenesis, microglia phagocytic activity is compromised, leading to senile plaque formation and accumulation [420]. Moreover, Aβ represents a crucial NLRP3 inflammasome activator in AD patients contributing to amyloidosis and neuropathology. The activation of this process concerns not only microglial cells but also circulating peripheral monocytes [359].

The degradation of extracellular Aβ by microglia can also occur through autophagy which is important for the regulation of Aβ-mediated NLRP3 inflammasome activation. Alterations of autophagy have been involved in the pathogenesis of AD and it has also been largely reported that neuroinflammation can downregulate autophagy pathway [505]. There is an interplay between autophagy and NLRP3-inflammasome activation, autophagy can interfere with multiple steps of inflammasome activation, through the removal of its activators, clearing its protein components, and mediating the downstream cytokines degradation [360]. Our results obtained in macrophage-derived THP-1 cells have shown that, D4T downregulated the expression of NLRP3 proteins-related mRNA and also IL-18 production and release. Moreover, D4T together with its dampening effect on NLRP3 activation, significantly reduced p-ERK and p-AKT phosphorylation [490]. This modulation might upregulate macroautophagy, dampening mTOR inhibitory signals. Noteworthy, D4T significantly upregulated Beclin-1, Lamp2A, and p70S6K expression levels, suggesting that this compound stimulates autophagy-mediated Aβ clearing acting on different catabolic pathways, possibly as a consequence of its ability to hamper NLRP3 inflammasome activation [490]. Therefore, we analyzed D4T role on gene expression of the glutamate transporter EAAT1, NLRP3 and β-arrestin-1 to evaluate D4T regulation on NLRP3 and to investigate its possible effect on molecular mechanisms that are impaired in AD. In fact, EAAT1 expression is impaired in AD patients' brains, where it is mainly expressed, but it is also present in peripheral cells [521]. The expression of EAAT1 is upstream regulated by the ERK/CREB pathway through their phosphorylation [95], albeit the modulation observed in phospho-ERK1/2 signaling pathway upon D4T exposure, no significant effects have been reported in EAAT1 mRNA levels. At this regard, we propose to investigate whether D4T could modulate EAAT1 protein levels through translational or post-translational modifications.

Noteworthy, D4T reduced NLRP3 gene expression in presence of cytotoxic stimuli, suggesting that it might prevents the Aβ induced inflammation acting on NLRP3 transcription.

In order to understand the mechanism by which D4T dampened NLRP3 activation, we analyzed β-arrestin-1 gene expression. β-arrestin-1 is a member of multifunctional proteins, involved in various signaling pathways [543], and it also plays an important role in the assembly and activation of NLRP3 inflammasome in condition of inflammatory stimulation [544]. It has been hypothesized that activation of the α7 nicotinic acetylcholine receptor (α7nAChR) inhibits NLRP3 inflammasome activation by regulating β-arrestin-1 [519,545]. To verify whether D4T dampened NLRP3 activation through this pathway, we analysed β-arrestin-

1 mRNA levels. In line with the literature, our results have been shown that β-arrestin-1 transcription is significantly induced by Aβ stimulation, while it decreased upon D4T exposure, suggesting that D4T probably dampened inflammasome activation by decreasing β-arrestin-1. Therefore, we can hypothesize that D4T could also modulate the nicotinic acetylcholine receptors, which are well-known to be compromised in AD. In order to confirm our hypothesis, we propose to extend our analysis on β-arrestin-1 protein levels and the expression of the α7nAChR.

Recently we have described that D4T counteracts inflammasome activation preventing active caspase-1 production and release, but it did not restore microglial Aβ phagocytosis [490]. Starting from these findings, we investigated the role of D4T on Ras/MAPK and GSK3β/PI3K pathways and autophagy modulation, in $A\beta_{42}$ treated PBMC-derived monocytes from recruited AD patients.

First of all, we focused our attention on p38 SAPK, ERK1/2 and PI3K/AKT signaling pathways, which are deregulated in AD. p38 SAPK is activated in macrophages and neutrophils extracellular inflammatory mediators such as cytokines, chemokines, and also LPS, regulating the progression of the cell cycle and promoting pyroptosis [546]. Moreover, p38 signaling is induced in AD patients by elevated levels of IL-1β leading to tau hyperphosphorylation [271].

In line with these findings, our results showed increasing p-p38 levels following LPS priming and Aβ stimulation in PBMC-derived monocytes, whereas D4T can prevent the toxic effect due to Aβ exposure significantly reducing phosphorylation of p38 in HC and AD monocytes as well. These observations suggest that D4T-mediated downregulation on p-p38 could be beneficial in reducing neuroinflammation.

Then, we analyzed D4T modulation on ERK1/2 and PI3K/AKT signaling pathways which upstream regulate autophagy, a complex cellular process, which mediates the degradation of intracellular components and it plays an important role in regulation of innate and adaptive immunity, modulating caspase-1 mediated immune response [547]. Moreover, autophagic process is essential for homeostasis and development of the CNS, clearing expired organelles, and damaged intracytoplasmic contents in neurons, which otherwise could lead neuronal activity impairment [339]. Three major forms of autophagy have been described: micro-autophagy, macroautophagy, and CMA which crosstalk with each other, in order to eliminate aberrant proteins and modulating apoptosis [548]. Autophagy counteract the apoptotic cell death by promoting cell survival through the removal of damaged organelles that are source of cytotoxic ROS or thereby the catabolism of macromolecules and degrading misfolded proteins aggregates [549].

Several studies have been reported that neuroinflammation can hamper autophagy pathway and its malfunction can lead to AD progression [344,508] because of Aβ peptides accumulation in autophagosomes of dystrophic neurites. Thus, it can lead to a further accumulation of intracellular Aβ plaques and neurodegeneration [550,551]. Neurons engulfed by Aβ plaques are surrounded by reactive microglia which

release pro-inflammatory cytokines. This can modify the progression of the disease, demonstrating a key involvement of neuroinflammation in AD [12].

ERK1/2 is involved in the regulation of autophagy, modulating the mTOR pathway and phosphorylation of p70S6 kinase, moreover PI3K/AKT phosphorylates mTOR, leading to inhibition of macroautophagy. P-AKT regulates CMA also, inducing phosphorylation of intermediate proteins, which hamper the oligomerization of LAMP2A, a channel protein which mediate the translocation of the proteins within lysosome for degradation [552]. We observed that D4T up-regulated both p-ERK and p-AKT in HC and AD PBMC-derived monocytes following cytotoxic stimuli, suggesting that D4T might modulate also their downstream targets.

Therefore, we investigated D4T impact on Beclin-1, LAMP2A, p-p70S6K and its nuclear isoform p-p85S6K. Our results have been shown that D4T upregulated both phospho-p70S6K isoforms and LAMP2A expression, while a reduced modulation has been detected on Beclin-1. Given the fact that p-ERK1/2 and p-AKT exert an inhibitory effect on autophagy, we expected a reduction on Beclin-1 and LAMP2A expression upon D4T treatment, otherwise the results herein shown the contrary effect. We assume that D4T up-regulates the downstream ERK1/2 and PI3K/AKT pathways, it is mainly effective on stimulating chaperone-mediated autophagy related target instead of macroautophagy.

Thereafter, we analyzed the D4T impact on apoptosis, investigating the expression of Bax, which is upstream regulated by AKT. Apoptosis is a highly regulated process that promotes death and phagocytic clearance of damaged cells. When Bax is activated, oligomerize with BAK and permeabilize the mitochondrial membrane facilitating cytochrome c release and inducing apoptotic cell death [553]. Furthermore, a possible mechanism of p38 SAPK mediated apoptosis has been reported in AD. Activated p38 can phosphorylate Thr residue of Bax leading to its subsequent translocation to mitochondria and apoptosis of neuronal cells [518].

Since, our previous results showed that D4T significantly reduced p38 activation, we expected a consequent reduction in Bax expression too, instead D4T increased Bax expression in monocytes from recruited AD patients, while any modulations are detected in HC ones. We assume that in AD patients, apoptotic mechanisms could be impaired, thus cells may do not respond properly to acute stress stimuli, such as Aβ exposure. We hypothesized that D4T could facilitate apoptosis to counteract Aβ cytotoxicity, leading to death the damaged neurons.

ERK1/2 and PI3K/AKT signaling pathways, upstream regulate also CREB activation [220], which plays many different roles in immune function promoting anti-inflammatory immune responses, through the inhibition of NF-kB activity and the induction of anti-inflammatory cytokines [308], while in the CNS, CREB has a well-known role in neuronal plasticity and memory functions. Low levels of p-CREB have been reported in the prefrontal cortex and in the hippocampus of postmortem AD brains and the same

alteration have been found also in PBMCs [314]. Noteworthy, our data have shown that D4T increased pCREB in monocytes from HC and AD patients, probably as a consequence of p-ERK and p-AKT increasing due to this compound. Based on these results, we assume that D4T could attenuate the inflammatory response on the peripheral level and might counteract the alterations on pCREB observed in AD.

Beyond autophagy and apoptosis, we have investigated possible modulations of D4T on phagocytosis, evaluating its impact on TREM2 expression. TREM2 is a cell membrane receptor expressed by monocyte derived dendritic cells and microglia in the CNS [427], and it plays a pivotal role in microglia migration, cytokine release, phagocytosis and proliferation [428]. Previous studies in macrophage-derived THP-1 cells have been described that D4T counteracts inflammasome activation, but it did not restore microglial Aβ phagocytosis [490]. In line with these data, our results have shown that D4T slightly reduced TREM2 expression in monocytes from AD patients, suggesting that this compound is not effective in mediating Aβ clearing through phagocytosis, which is compromised in AD. However, we hypothesize a role of D4T on inducing Aβ clearing through autophagy, in particular this compound seems to be more effective on CMA rather than macroautophagy.

7.4 Investigation of NLRP3 inflammasome pathway activation in Aβ-related pathology

Several lines of evidence have found a mutual interaction of innate immune mechanisms and neurodegeneration [374,554]. In particular, the NLRP3 inflammasomes activation in microglia, play a key role in mediating the innate immune reaction in AD [463], since is activated by different size of Aβ aggregates and also by lower molecular weight Aβ oligomers and protofibrils [464]. Microglia can sense Aβ, and high presence of oligomers in the AD brain, can result in the increased of Aβ phagocytosis by the microglia and by the recruited peripheral monocytes [555]. This chronic inflammation state, alters microglial clearance capacity and leads to the engulfment of these cells, favoring the Aβ accumulation and deposition in plaques in a self-feeding pathogenic loop [406,524].

For these reasons, in the third part of this study, we investigated the NLRP3 inflammasome pathway activation in AD using THP-1 human monocytes cells and monocytes-derived microglial cells (MDMIs) obtained from recruited HC subjects and AD patients. First of all, we performed cell signaling and functional studies in THP-1 cells, to set up the best experimental conditions in order to analyze NLRP3 inflammasome activation. THP1 cells constitutively express NLRP3, and its activation occurs in response to well-known NLRP3 activation stimuli including LPS and Nigericin, which subsequently lead to extracellular release of IL-1β [556].

In line with these data, we analyzed the expression of NLRP3, ASC, caspase-1 and its active form caspase-1 p20, in THP-1 cells following 3h priming with LPS and 1h stimulation with 10μM and 20μM Nigericin, to

induce a strong inflammasome activation. We also quantified IL-1β release in supernatant as a result of NLRP3 activation. Our results have shown an increasing dose dependent trend of NLRP3, ASC, Caspase-1 and the cleaved form p20 protein expression upon LPS priming and stimulation with Nigericin on cell lysates and proteins precipitated from the supernatants. Moreover, we observed a strong dose dependent IL-1β release on the supernatants suggesting a strong NLRP3 inflammasome activation.
THP-1 cells can eliminate Aβ through phagocytosis [557]. We tested Aβ-FAM and Aβ-TAMRA phagocytosis in order to identify the best fluorescent tagged peptide in pre-incubated THP-1 cells for 30min with Cytochalasin D. Functionally, cytochalasin D inhibits microfilament function and polymerization by blocking actin monomer addition [558] and it is used as a phagocytosis inhibitor [559]. Thereafter, THP-1 cells were exposed to Aβ-FAM, Aβ-TAMRA peptide and pHRodo beads [560], as a positive control of phagocytosis for 6h, 4h, 2h and 30min. We observed an increase of Aβ-FAM phagocytosis in time dependent manner until 4h, after 6h THP-1 may started to degrade the peptide. While THP-1 cells did not seem engulf Aβ-TAMRA. We hypothesized that the lower uptake of Aβ-TAMRA could be due to the different chemical composition of the fluorescent reporter. Given that, Aβ-FAM was selected for further phagocytosis experiments in MDMIs.

Microglial cells are the resident immune cells in the central nervous system, and play a pivotal role, not only in neurogenesis, neuronal plasticity, and regeneration but also in immune defense [383]. As a matter of fact, that is very hard obtaining living brain cells including microglial cells from human brains. To solve this limitation, we derived microglia-like cells from human monocytes, isolated after blood withdrawal from recruited AD patients and HC. We observed that monocytes when cultured with, GM-CSF [561] and IL-34 [562], acquired a ramified morphology, typical of microglia. After 14 days of differentiation, we tested whether the monocytes derived microglia-like cells displayed a myeloid phenotype, analyzing the expression of surface and intracellular microglial markers. Our results have shown that MDMIs expressed CD11b, CD45, CD14, CD16, HLA-DR, typical myeloid surface markers [563]. Microglia-like cells displayed also CD43 and CD34 hematopoietic markers, the intracellular scavenger receptors CD36 and CD68, and chemokine receptors such as CCR2, CCR4, CX3CR1, suggesting that MDMIs have many characterization of microglial cells [564]. Since, a single microglial specific marker is still unknow, it is not easy to distinguish microglia from macrophages or other myeloid cells [529]. Hence, we characterized MDMIs for markers which are mainly expressed in microglia, in particular TREM2, TMEM119, IBA1, Pu.1 and P2YR12 [565–567]. We observed that MDMIs exhibited all of these markers, suggesting that they have a myeloid phenotype. Noteworthy, MDMIs expressed TMEM119 and P2YR12. TMEM119 is a cell-surface protein specifically expressed by parenchymal myeloid cells in the CNS and it is the best promising candidate for human microglial marker [395]. P2RY12 is a purinergic receptor typically expressed in homeostatic or non-activated microglia [568]. Taken together these findings, suggest that monocytes derived microglial cells reflected a myeloid

phenotype, hance they have revealed to be an attractive in vitro model of human adult microglia, suitable for in vitro microglial cell studies.

The microglia-specific activation of the NLRP3 inflammasome is pivotal for AD pathogenesis, hence we analyzed NLRP3 inflammasome pathway activation in MDMIs, analyzing the expression of NLRP3-related proteins and the subsequent pro-inflammatory cytokines release. At this purpose, MDMIs were primed for 3h with LPS and stimulated with ATP for 1h and Aβ for 24h with or without CRID3, a selective NLRP3 inflammasome inhibitor. Several lines of evidence have demonstrated the increased expression of NLRP3 inflammasome and the subsequent production of IL-1β and IL-18 pro-inflammatory cytokines in AD brains [466,569]. In line with these data, our results have shown higher pro-inflammatory cytokines release in supernatants collected from AD MDMIs compared to HC, upon LPS priming and ATP and Aβ stimulation, indicating that MDMIs might reflect the same pro-inflammatory conditions found in AD brains. Furthermore,
IL-1β and IL-18 production was increased both in Aβ and ATP stimulated MDMIs from AD patients and HC following LPS priming compared to untreated cells, while CRID3 treatment could counteract pro-inflammatory cytokines release in HC and also AD MDMIs, suggesting CRID3 inhibited the Aβ-induced immune response. Regarding the expression of NLRP3-related proteins, our results showed an increasing trend in caspase-1, its cleaved form caspase-1 p20 and ASC following LPS priming and ATP and Aβ stimulation, whereas CRID3 could reduce ASC protein expression due to ATP and Aβ stimulation in MDMIs from AD patients. Unfortunately, there are some limitations in this study due to the low sample size and for this reason it is not possible to obtain statistical significances. The data herein shown only reveals a decreasing trend of NLRP3-related proteins expression upon CRID3 treatment.
Exposure of microglia to Aβ triggers two distinct responses: phagocytosis and clearance of the peptide, but NLRP3 activation negatively affects the microglial clearance functions [570]. Recently, have been reported that the inhibition of NLRP3 inflammasome due to CRID3 exposure, increases the phagocytic ability of microglia, reduces Aβ accumulation and has a beneficial effect on cognitive functions in AD mouse model [485]. We analyzed the modulation of this processes in MDMIs from AD patients, inhibiting inflammasome activation with CRID3 and pre-incubating MDMIs for 30 min with Cytochalasin D, to inhibit phagocytosis. Thereafter, MDMIs were exposed to Aβ-FAM peptide for 6h, 4h, 2h, 1h and 30 minutes. Our results have shown that in microglia-like cells, Aβ uptake increased in time-dependent manner, while Cytochalasin D pretreatment dampened Aβ phagocytosis. Degradation of the peptide was analyzed after 17h, and we observed that only a small fraction of Aβ-FAM was still inside the cells upon a long time of exposure to the peptide, suggesting that MDMIs are able to clear Aβ and the clearance rate depends on the amount of the peptide which they have previously up-taken. Nevertheless, any significant effect due to CRID3 on Aβ uptake and clearance has been detected. We hypothesized that the acute

exposure to CRID3 might not be enough to induce a significant effect on Aβ phagocytosis and degradation pathways in AD MDMIs. Moreover, because of the small sample size, we must consider these results as preliminary data, further investigations are needed to clarify the role of CRID3 on Aβ phagocytosis. Furthermore, it would be interesting investigate the impact of CRID3 on other catabolic pathways involved in Aβ degradation such as autophagy and ubiquitin-proteasome system.

Several data have reported the efficacy of CRID3 in blocking inflammasome activation and the subsequent production and release of pro-inflammatory mediators in AD animal models [464,484,487]. Our data confirmed these evidences in human monocytes-derived microglia cells for the first time. For these reasons, MDMIs have revealed to be an attractive in vitro model of human adult microglia, suitable for in vitro microglial cell studies. However, our study presented some limitations that should be considered. MDMIs cells were differentiated from peripheral blood monocytes and may be different from microglia in the brain. Nevertheless, our data revealed that MDMIs may reflect microglial phenotype and functions. For these reasons, MDMIs cells represent an interesting and useful tool for generating microglia-like cells quickly and easily from human monocytes.

Chapter 8

Conclusions

Our study has investigated the molecular mechanisms involved in AD and in neuroinflammation, using peripheral ex vivo cellular models, in order to characterize the complex interactions among Aβ oligomers and intracellular signaling pathways. Furthermore, we investigated the potential protective role of natural and synthetic drugs against neuroinflammation and Aβ toxicity, to understand whether these compounds could be a suitable strategy for AD therapy.

Exploring Ras/MAPK and PI3K/AKT signaling pathways in fibroblasts from recruited subjects, we demonstrated that there is a close relationship between p-AKT and p-ERK levels and the disease severity and these signaling pathways could be suitable to check the disease molecular mechanisms in fibroblasts from AD and MCI patients, and p-ERK could help to discriminate between converter and non-converter MCI.

The correlation between αAPP and ERK phosphorylation in HC fibroblasts demonstrated a direct involvement of ERK1/2 pathway in αAPP metabolism, but this correlation is discontinued during AD progression. The hyperactivation of PI3K/AKT signaling pathway, as a consequence, lead to increased expression of its downstream targets, p70S6K and Bax. For these reasons AKT seems to play a more specific role in tau phosphorylation, autophagy and apoptosis regulation. Moreover, we underlined that the regulation of mTOR signaling in AD also has a kay role in fibroblasts, which are a useful ex vivo model for biomolecular and pharmacological research.

Targeting toxic oligomers by dis-aggregating agents could be an attractive strategy to prevent Aβ aggregation. In particular, interest in nutraceuticals, functional foods and the potential benefits of natural compounds consumption, such as coffee [214] and Hop have greatly increased in recent years. In particular, we investigated the potential protective role of hop extracts against Aβ toxicity. Our findings suggested that Hop modulates specific signaling pathway activated in response to cytotoxic stimuli and involved in the disease pathogenesis, suggesting that Hop has potential to be a promising prophylactic strategy against AD. Starting from these data, could be interesting to investigate the Hop extracts role on ERK1/2 and PI3K/AKT downstream targets, autophagy and inflammation related pathways, to clarify whether interventions aimed at strengthening mitochondrial activity and stimulating macroautophagy and CMA could therefore prevent the neurodegenerative process in AD.

Adult human fibroblasts could be reprogrammed to obtain human induced pluripotent stem cells (iPSCs) and differentiated into cell-types of their relevant diseases [571]. Fibroblasts provide a useful cellular model for studying AD, since these cells have been shown to express a broad spectrum of AD-related cellular abnormalities. For these reasons, fibroblasts have a great potential for research and clinical purposes by providing a useful tool for the development of cell models for neurodegenerative diseases.

Regarding the influence of peripheral inflammation in AD, we examined the involvement of NLRP3 inflammasome activation on Ras and GSK3/PI3K pathways and autophagy modulation in PBMC-derived

monocytes from AD patients and healthy subjects upon D4T treatment, that reduces NLRP3 inflammasome activation.

Our findings revealed that D4T, modulates Ras/MAPK and PI3K/AKT signaling pathways, through which it prevents inflammasome activation, it also up-regulates CMA, CREB phosphorylation and modulates Bax, a pro-apoptotic protein.

Since D4T can hamper NLRP3 inflammasome activation, and as a consequence the neuroinflammation, we explored the involvement of D4T on catabolic pathways among them, autophagy and phagocytosis, and we observed that D4T induces the intracellular Aβ clearing processes. Further investigations will be focused on the ubiquitin-proteasome system, another catabolic pathway which plays a pivotal role on intracellular Aβ clearance. We will perform further investigations to explore D4T molecular mechanisms and to better understand its involvement in Aβ catabolism. We propose to investigate D4T impact on the regulation of gene expression of macroautophagy and CMA related targets, taken in exam in this study, to assess whether D4T acts on these targets at transcriptional or post-transcriptional level. We propose to expand our analysis, investigating other autophagy regulators such as LC3 and p62, which mediate the catabolism of polyubiquitinated protein aggregates thereby autophagy [338]; and also hsc70, the main carrier protein of CMA together with LAMP2A.

Preliminary data generated in our laboratory have shown that D4T increased caspase-8 expression in AD PBMC-derived monocytes in presence of cytotoxic stimuli, strengthening the hypothesis of a possible D4T role in apoptosis induction. We assume that the induction of apoptosis due to D4T could be protective, leading to death the damaged cells by Aβ exposure.

However, in light with our results D4T seems to be a promising approach in AD therapy, but further analysis are required to clarify its possible use on a clinical scenario, possibly using AD animal models to assess the D4T impact on cognitive function and to investigate its role on Aβ catabolism “in vivo”.

To date efficient treatments that aim at preventing or mitigating the cognitive deficits and AD progression are still lacking. One of the main reasons is the difficulty in obtaining relevant models that show all aspects of AD pathology. Noteworthy, sporadic AD patients, often present comorbidities that are risk or promoter factors of AD, which are not easily replicable in animal models. Moreover, it is very hard obtaining cells from the human brain. In order to characterize the microglia-specific NLRP3 inflammasome activation, we used an in vitro model composed of human AD and HC monocytes differentiated into microglial-like cells. We demonstrated that these microglia-like cells maintain myeloid functional phenotypes including Aβ peptide phagocytosis and induction of pro-inflammatory cytokines release in response to inflammatory stimuli. In the future we propose to examine the genetic profile of MDMI cells analyzing the expression of microglia-specific genes.

Furthermore, we explored the role of the NLRP3 inhibitor CRID3, to investigate the effects of NLRP3 inflammasome inhibition through another pathway. Compared to D4T, which inhibits NLRP3 activation blocking the purinergic receptor P2X7R, CRID3 is a selective and direct NLRP3 inhibitor and we demonstrated that this synthetic drug is able of significantly reducing NLRP3 inflammasome activation and the subsequent downstream production of proinflammatory cytokines in both MDMIs from healthy subjects and AD patients. MDMIs share many features with human microglia and provide a useful tool for exploration of several biological behaviors of human microglia in neurodegenerative disorders. For these reasons, monocytes-derived microglia cells could be a useful ex vivo model for exploring microglia function and dysfunction. MDMI cells are differentiated from patient's monocytes and they could be useful in drug screening, in analysis of biochemical or functional changes in response to drugs and in setting up a personalized therapy.

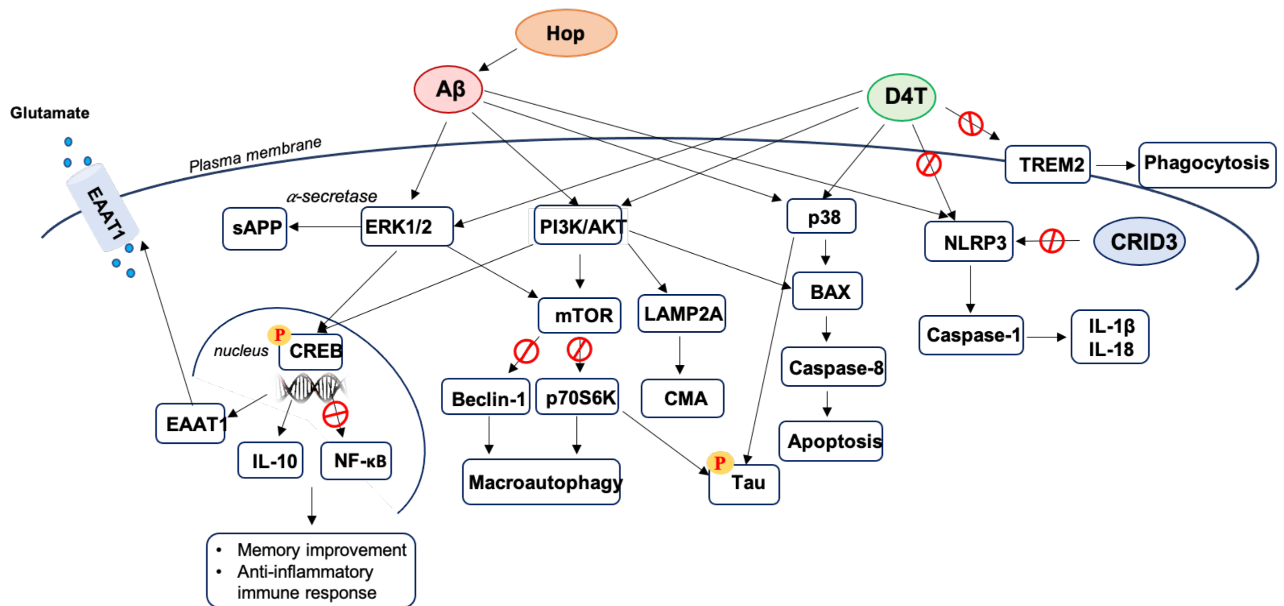
However, additional investigations are required to better characterize NLRP3 inflammasome activation pathway. NLRP3 inflammasome activation requires the oligomerization of the adaptor protein ASC into a large 'ASC speck' complex via CARD-CARD domains. This is a crucial step for NLRP3 inflammasome assembly and activation since it leads to caspase-1 activation, which subsequently cleaves pro-IL-1β to the mature and active form IL-1β [572]. We propose to investigate more deeply the NLRP3 inflammasome activation pathway and the mechanism of action of CRID3, analyzing the impact of this drug on NLRP3 downstream targets including ASC speck formation and caspase-1 activity in MDMIs.

Activated caspases can mediate the cleavage of downstream gasdermin D (GSDMD), which forms oligomers, on the cell plasma membrane leading cells to pyroptosis [573], a lytic form of cell death that is important for immune defense, and also GSDMD plays a key role in IL-1β release [455]. Despite the importance of gasdermin in pyroptosis and inflammation, the mechanisms of action of the gasdermin family are still unknown [574].

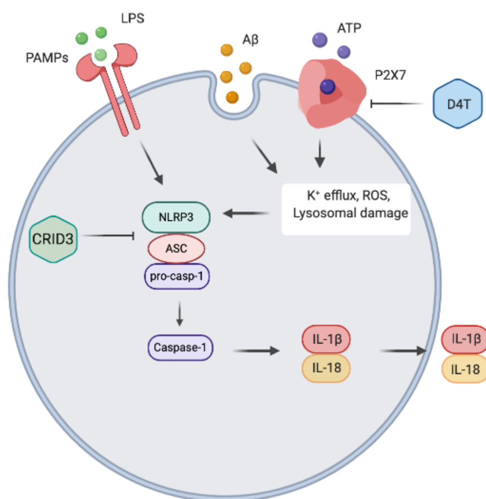
It will be interesting investigate the impact of CRID3 on GSDMD activation, to better understand its mechanism of action and to verify whether NLRP3 inhibition by this compound could subsequently hinder GSDMD activation and pyroptosis.

Furthermore, due to the small sample size, this study exhibits some limitations. Unfortunately, the current pandemic situation has restricted patients' recruitment, and therefore it is required to proceed in the future to increase the number of AD and age-matched HC samples to compare the different responses to NLRP3 activation and better understand whether new therapeutic strategies, which in our ex-vivo model inhibit or prevent the NLRP3 inflammasome assembly and activation, could be an effective AD therapy. In addiction further investigations are needed to better understand whether CRID3 treatment could prevent or counteract the alterations on microglial phagocytosis due to inflammasome activation. We observed that D4T does not modulate phagocytosis in both macrophages-derived THP-1 cells and PBMC-

derived monocytes from recruited subjects, while this compound seems to mediate Aβ clearing through autophagy. We propose to investigate the role of CRID3 on other pathways involved in Aβ degradation such as macroautophagy, CMA and ubiquitin-proteasome system, to compare the effects between the two different NLRP3 inhibitors on Aβ clearance.



Intracellular signaling pathways and new strategies against Aβ cytotoxicity and neuroinflammation



Representative image of NLRP3 inflammasome activation pathway.

Chapter 9

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