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BEHAVIOURAL AND PSYCHOLOGICAL SYMPTOMS OF DEMENTIA: ROLE OF THE IMMUNE RESPONSE

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Quello che ho capito, crescendo, è che

tutto ciò che puoi fare per i tuoi genitori

non eguaglierà mai quello che loro hanno fatto per te.

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Chapter 1

Alzheimer's Disease

1.1 Disease Overview

Alzheimer's Disease (AD) represents the most common form of dementia among elderly, affecting around 10 and 40% of people over the age of 65 and 85, respectively and it is a progressive, irreversible neurodegenerative disorder, ultimately leading to death. It is clinically characterized by age-related memory loss and cognitive impairment, often accompanied by personality changes[1–3]. These damages are closely correlated to brain atrophy and cortical thinning[4,5], together with neuronal injury in areas where synaptic disruption, gliosis and loss of information integration are seen as well[6]. Even though alterations have been found in the frontal, parietal and occipital lobes[7], one of the most compromised regions is the hippocampus, which is fundamental for mnemonic processes[8]. Indeed, episodic and spatial memory are damaged at first, followed by executive functions in later stages of the disease[9,10].

From a biological point of view, this disease is characterized by extracellular amyloid plaques, containing $A\beta_{40}$ as well as $A\beta_{42}$ peptides, and intra-neuronal neurofibrillary tangles, which consist of hyper-phosphorylated tau protein. Despite being always present in demented patients, they are not considered absolute markers for the pathology, since they have both been found also in aged healthy individuals. Moreover, given that the disease progresses slowly, AD diagnosis is often delayed until pathological symptoms become evident[11], making clinical interventions more difficult. However, neither reduced hippocampal volume nor is not always positively correlated to cognitive impairment, since it could also be a normal part of ageing[12].

1.2 Epidemiology

AD estimated prevalence in 2015 was 44 million people all over the world and this number is expected to double by 2050[13]. According to the 2016 CENSIS-AIMA report, 600,000 AD patients are present in Italy and 50,000 new cases are diagnosed every year. These figures are destined to rise in an exponential manner in the near future and, for this reason, finding a cure is essential to manage this severe disease that is quickly spreading worldwide[14] and that represents a high burden not only for the patients and their families but also for the healthcare system. In the Italian population between 65 and 84 years old, AD incidence is 6.6 affected people every 1000 inhabitants per year and its prevalence is 4.4%[15]. However, this number increases with age,

reaching 8% in people between 80 and 84 years old and it is higher in females, most likely because they live longer than males[15] but also due to biological factors and lifestyle experiences[1].

1.3 Pathological forms

There are two different forms of this pathology, the sporadic or late-onset AD (LOAD), accounting for 95% of all cases and depending on multiple factors, such as environment and genetic predisposition[9,16] and the familial or early-onset AD (EOAD), typical of young individuals (normally under 65 years old) and linked to autosomal dominant missense mutations in three different genes[17,18].

➢ Early-Onset AD

The first mutation is located on chromosome 21 and encodes the amyloid precursor protein (APP), which not only might regulate calcium and metal ions homeostasis, cholesterol binding and cell growth but could also stimulate fibroblasts proliferation and cell adhesion[19]. Differently, the second and the third gene, situated on chromosome 14 and 1, respectively, are responsible for the production of presenilin-1 (PSEN-1) and presenilin-2 (PSEN-2), which are subunits of the γ-secretase enzymatic core and have a role in autophagy-mediated degradation of protein aggregates[20]. Most of the mutations affecting these proteins are considered causative, because they lead to an impairment in the amyloidogenic pathway, inducing an increased accumulation of $A\beta_{42}$[21].

➢ Late-Onset AD

On the contrary, the risk factors for LOAD can be either susceptibility genes or environmental elements[22] and it has been proposed that they could have an additive effect, so that the more they are in an individual, the worse will be the developed pathology[22]. The strongest genetic component affecting AD manifestation is represented by apolipoproteinE (ApoE), which is 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[23,24]. It binds and transports lipids through both lymphatic and circulatory system, so that its functional alterations result in increased

plasma levels of cholesterol and triglycerides[25]. Moreover, its gene displays three different alleles (ε2, ε3 and ε4)[24], which influence the shape of ApoE protein and its ability to facilitate Aβ clearance, a process that strictly depends on the bond between amyloid and ApoE itself[26]. Indeed, homozygous or heterozygous subjects for ApoEε4, which show the worst efficacy in transporting Aβ[27] and the most abundant deposition of this peptide, together with glucose hypometabolism, mitochondrial anomalies and oxidative stress[24], have a very higher risk of developing AD compared to non-carriers[28,29]. Also, the presence of this allele might be helpful to identify diseased individuals, since their brain functional connectivity shows an altered resting state, which could be early detected with fMRI[30]. On the other hand, the lower prevalence of ApoEε2 in demented patients highlights its protective role in the pathology[28,31].

However, thanks to genome-wide association studies (GWASs), many other genes have been identified as potential risk factors for developing AD and many of them, such as CD33, TREM2, ABCA7, CLU, CR1, CD2AP, MS4A and MEF2C, are related to the immune system[25].

Beyond genetics, LOAD has also strong environmental components that could facilitate its onset and the best-known among them is ageing, which doubles the risk of developing this pathology every 5 years after age 65[32]. Apart from that, another recognized predisposing factor is hypercholesterolemia. Indeed, AD patients usually display high plasma levels of both cholesterol and its oxidation derivatives oxysterols[33] It has been demonstrated that APP metabolism and trafficking, secretases activity and Aβ synthesis are influenced by cholesterol distribution within the membranes and changes in the levels of this molecule cause lipid rafts impairment, which negatively influences APP cleavage by γ-secretase in these areas[33]. Thus, inhibition of cholesterol synthesis have been shown to reduce the activity of this enzyme and to increase α-secretase one, promoting neuroprotection[34].

Instead, a different link has been found between AD and obesity, which alters brain homeostasis causing inflammation and oxidative stress[35]. Indeed, this food disorder is correlated to an up-regulation of pro-inflammatory adipokines and a down-regulation of anti-inflammatory factors, such as BDNF[36]. This imbalance induces neuroinflammatory processes as well as impaired neurogenesis and synaptic plasticity, promoting neurodegeneration[37]. Lastly, other predisposing factors to AD pathology seem to be hyperhomocysteinemia and hypertension, which has been linked to brain atrophy as well as NFTs formation.

1.4 Aetiology

To date, AD aetiology has not been clearly elucidated yet, given that this disease is very complex and multifactorial. Thus, it is more likely due to a combination of different factors rather than to one single cause. However, several theories have been proposed, in the attempt to explain its pathogenesis.

➢ Amyloid cascade hypothesis

The most popular is the amyloid cascade hypothesis, which asserts that AD originates from a series of anomalies in both the productive and secretive pathway of APP. This is a transmembrane glycoprotein of type I expressed in neuronal tissues, which mostly undergoes a non-amyloidogenic consecutive cleavage, before by α-secretases such as ADAM10, ADAM17 or ADAM19[38,39] and after by γ-secretase, which is a heteromeric complex containing presenilin-1 (PSEN-1) or presenilin-2 (PSEN-2), nicastrin (NCSTN), anterior pharynx defensive phenotype 1 (APH-1) and PS-enhancer-2 (PEN-2)[40]. The first cut generates a soluble APPα (sAPPα) peptide, endowed with trophic and neuroprotective functions and a C83 carboxi-terminal fragment, whereas the second one produces a small AICD portion and a p3-peptide[41,42].

However, a second amyloidogenic processing pathway for APP exists. In this case, the first cleavage is performed by a β-secretase enzyme, such as BACE1, which generates a soluble APPβ (sAPPβ) peptide and a C99 carboxyl fragment, whereas the second γ-secretase-dependent cut produces AICD and amyloid-β[43]. This poorly soluble molecule is further secreted through exocytosis from either the endoplasmic reticulum or the endosomal-lysosomal system in the interstitial fluid[44] and, once in the extracellular space, it is prone to aggregate and accumulate in plaques[45].

It is possible to identify several amyloid peptides that differ for their length and, among them, the most abundant is normally $A\beta_{40}$[46]. Nevertheless, senile plaques mainly contain $A\beta_{42}$, which is not only easier to aggregate but also far more toxic than its shorter form and is greatly produced in AD, generating a higher $A\beta_{42}/A\beta_{40}$ ratio that has been linked to LOAD[47]. However, the excessive amount of this longer peptide has been shown to depend on mutations in the APP gene in EOAD as well, even though the total Aβ amount seems unchanged.

The amyloid peptide is resistant to proteolytic degradation and the amino acids sequence between position 25 and 35 seems to be the most neurotoxic, even though the mechanisms underlying this effect have not been fully understood yet[48,49]. Even though this molecule is physiologically cleared from the brain, it often misfolds, acquiring a β-sheet configuration that spontaneously tends to dimerize[50]. In a similar way, these dimers form oligomers, which might assume a fibrillar or a non-fibrillar conformation[51] and, interestingly, appear to be the most toxic species. Lastly, their accumulation originate plaques, which have been recently shown to be biologically inert[41,52].

It is believed that oligomers trigger synaptic failure and neurodegeneration[53,54], inflammation [55], oxidative stress[56–58], membrane increased permeability and thinning[59,60], cholinergic receptors impairment[61] NMDA receptors over-stimulation[62] and DNA modifications[63]. Moreover, they can generate hydrogen peroxide and hydroxyl radicals that cause lipid peroxidation, impairing ATPases and glucose transporters, ultimately leading to an imbalance in calcium and energy homeostasis[64–66]. On the other hand, they have been shown to impair mitochondrial electron transport chain, promoting the dysfunction of these organelles and leading to cell death[67–69]. However, all these damaging effects seem to depend on a critical oligomers concentration, which acts as a threshold to originate the down-stream signalling, together with hippocampal atrophy and dementia[70]. Nonetheless, plaques density has been demonstrated to correlate with the probability of MCI-to-AD progression but not with cognitive impairment in AD patients[71–75], thus amyloid aggregation does not seem the only pathogenic trigger.

Indeed, the ineffective clearance of this peptide might generate toxic species and has been thought to have a role in the disease as well. The proposed mechanism posits that cerebral Aβ concentrations increase because of ageing and due to the reduced expression of both lipoprotein receptor-related protein-1 (LRP-1), which is responsible for amyloid passage from brain to blood and Aβ degrading enzymes, such as neprilysin and insulin-degrading enzyme[76,77]. In support of this hypothesis, enhancing amyloid elimination in AD mouse models improved cognitive symptoms[78–80] and it has been demonstrated that age increases Aβ deposition but not its production, indicating that its clearance might be defective[58,81]. However, although this peptide has an undeniable role in AD pathogenesis, it is not believed to be the only aetiological factor[82].

➢ Cholinergic hypothesis

Indeed, the cholinergic hypothesis considers the role of acetylcholine in the onset of the disease, given that AD patients display a massive loss of cholinergic neurons in both the basal forebrain and the hippocampus, which are important regions for attention, learning and memory[83–85]. This damage is associated to alterations of choline uptake, acetylcholine release, expression of nicotinic and muscarinic receptors as well as axonal transport[86–88] and these deficits could be identified by the down-regulation of specific markers, such as acetyltransferase and acetylcholinesterase, which accompanies cognitive impairment onset[89].

Moreover, deficiencies in the cholinergic system might affect the glutamatergic one, which is also impaired in AD, since they strictly interact during neurotransmission[47,90]. Indeed, acetylcholine is thought to be protective against NMDA glutamate receptors hyperactivation[91–93], which could be also induced by amyloid-β[94,95] and is responsible for calcium overload, generation of free radicals and neuronal death[96,97]. All these processes have been observed in AD and have led to the development of pharmacological strategies targeting both the cholinergic and the glutamatergic system but, albeit their impaired neurotransmission has an important role in the pathology, it could not be considered its definitive cause[48,98].

➢ Tau hypothesis

As a secondary trigger of this disease, a third hypothesis points at tau protein, which is normally present in neuronal axons, where it is responsible for microtubules stabilization[99,100]. It is encoded by microtubule-associated protein tau (MAPT) gene, which could generate 6 isoforms that differ for the number of exons that their mRNAs contain[101] and it is physiologically highly soluble. However, due to a dysfunctional phosphorylation process, it becomes hyper-phosphorylated in AD and it reduces the interactions with microtubules, starting to aggregate with cytoskeletal proteins within neurons and leading to impaired axonal transport and mitochondrial damage[100,102–104]. As it happens for Aβ, tau deposition correlates with disease progression[105] and recruits soluble monomeric species to the already formed NFTs[106,107]. Moreover, the accumulated protein is acetylated, probably because of post-translational modifications occurring after fibrillization[108] and oligomers seem to be the most detrimental structures[109–111].

In spite of their similarities, the relationship between tau and Aβ has not been clearly elucidated yet[103,112] but most of the data claim that tauopathy derives from amyloid peptide accumulation[113–115]. On the contrary, some studies suggest that tau might mediate Aβ toxicity[116–118]. In support of this hypothesis, it has been demonstrated that neurons without tau gene are protected from Aβ-induced cell death[119].

➢ Mitochondrial and calcium hypothesis

Beyond amyloid peptide, tau protein and cholinergic neurons degeneration, which are still considered the main culprits in AD aetiology, some other processes have been shown to be severely impaired in the pathology and, thus, could represent causative factors. One of these is oxidative stress, which is a condition of lost balance between the production of reactive oxygen species (ROS) and anti-oxidants[120,121], leading to protein misfolding[122], lipid peroxidation and increased apoptosis[123]. These molecules are highly present in AD brains, together with alterations in the antioxidant enzymes[124]. It has been hypothesized that Aβ aggregation could cause ROS production, which in turn boost amyloid and tau accumulation but the precise mechanisms underlying this process have not been clarified yet[125,126].

In addition, oxidative stress deeply affects neuronal mitochondria, disrupting their functions and inducing caspases activation and cell death[127–131]. Interestingly, mitochondrial impairment as well as calcium dysregulation is also considered one of the possible aetiological factors in AD. Indeed, the cellular homeostasis of this vital ion is indispensable to survive and depends on the activity of endoplasmic reticulum, mitochondria and specific pumps[132,133]. These systems are damaged in AD neurons and their impairment causes a calcium overload, which is followed by energetic failure, Aβ and tau abnormal production and apoptosis[134–137]. Moreover, this process is stimulated by the amyloid peptide[138–140], which has also been demonstrated to impair mitochondrial biogenesis in pathological subjects[131,141]. On the other hand, the endosomal-lysosomal pathway seems to be dysfunctional too. Given that it regulates APP and tau metabolism[142], its defects might be responsible for AD pathogenesis[143,144].

➢ Neuroinflammatory and vascular hypothesis

Beyond these cellular mechanisms, another important trigger of the disease appears to be neuroinflammation, which strongly depends on activated microglia[17,145] and lastly, a vascular hypothesis has been theorized to explain BBB disruption as well as the reduction in cerebral blood flow observed in demented subjects[146].

Therefore, AD is a complex pathology not ascribable to one specific cause but rather depending on multiple factors, strictly connected one to each other. Thus, untangling this intricate scenario is mandatory, in order to develop efficient therapies to halt or reverse this tremendous disease.

1.5 Diagnosis

The pathology displays different degrees of severity, identified as a preclinical phase, an amnestic Mild Cognitive Impairment (aMCI) stage and, lastly, an overt dementia.

➢ Stages and clinical tests

The preclinical phase starts early in the lifetime of an individual and is totally asymptomatic at the beginning, even though amyloid has already started to accumulate and aggregate[147,148]. Later on, some CSF and brain biomarkers become detectable but still cognitive symptoms are lacking[5,149–151] until the end of the preclinical stage, when the impairment can be observed by means of specific tests such as Montreal Cognitive Assessment (MoCA), Alzheimer's Disease Assessment Scale Cognitive subscale (ADAS-Cog), Disability Assessment of Dementia (DAD) and Mini-Mental State Examination (MMSE)[152–154]. This one, in particular, establishes different categories based on the guidelines from the National Institute for Health and Care Excellence (NICE). So, according to the obtained score, it is possible to identify mild (score 21-26), moderate (score 15-20), moderately severe (score 10-14) and severe (score <10) dementia[155].

The MCI stage is characterized by reduced cognitive abilities, although the subject is still independent and active[156,157]. It manifests as a decline in memory, attention, language and executive functions, which can be more or less evident. To date, there are no validated tests to confirm this diagnosis, so that the patient has to rely on the experience of the clinician[158]. If

cognitive impairment is accompanied by the presence of CSF and imaging biomarkers, an underlying AD pathology is highly probable but, if these molecules are not detectable, most likely MCI symptoms are not related to AD[158].

The definitive diagnosis of this disease is possible post-mortem only, after the identification of its main hallmarks in the brain[159], so clinicians normally evaluate its putative presence using specific guidelines established by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) together with the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) criteria[160]. The main signs defining this disease include a strong cognitive impairment, which interferes with daily activities and behavioural anomalies not ascribable to other neuropsychiatric conditions[159].

- **Imaging**

However, the diagnosis of *probable* AD is hardly ever based just on these standards and often requires additional tools like neuroimaging techniques, which include Magnetic Resonance (MRI) and Positron Emission Tomography (PET)[161,162]. The first one provides high-resolution cerebral pictures to highlight abnormalities in anatomical and functional connectivity networks[30,158,163], whereas the second one allows the in vivo visualization of brain amyloid by means of the Pittsburgh compound B (PiB). Indeed, this molecule is very helpful in identifying Aβ spreading and burden, since it is able to detect its fibrillar form with high sensitivity[70,164], helping clinicians to stage AD[70,164]. In addition, PET might be used to track tau protein and its tangles, empowering the analysis[154] and, when fluoro-deoxyglucose (FDG) is employed as a tracer, this technique can reveal the lower glucose consumption of AD brains, compared to healthy ones[165,166]. Indeed, the pathology seems to be related also to metabolic disturbances, which might be explained with an impaired glucose transport through the blood-brain barrier (BBB) or a reduced energy demand for synaptic activity or a lesion in metabolic enzymes[167–169].

Despite their usefulness, brain imaging techniques are really expensive and require trained staff to run the machines, so that these examinations are not the best screening methods for wide groups of patients. For these reasons, clinical tests are often accompanied by biomarkers analysis, which can be very useful for an early identification of the disease, given that the pathological process begins decades before overt dementia onset[170–172].

➢ Biomarkers

Three proteins in the cerebrospinal fluid (CSF) are considered the best direct indicators of AD pathology available to date and, when combined, they give accuracy around 90%[158,159]. They are represented by $A\beta_{42}$ (associated with cortical amyloid), total tau (correlated to the intensity of neuronal degeneration) and phosphorylated tau in position 181 (p-tau181, marker of tangle pathology)[173]. Compared to healthy controls, individuals with AD exhibit low levels of the first one and high levels of the other two[174], which might also indicate MCI-to-AD progression[158,175–177]. Moreover, the tau(s)-to-$A\beta_{42}$ ratio has been shown to be predictive of cognitive decline in both MCI and mild-demented subjects[178–180].

However, in order to increase diagnostic accuracy, other CSF biomarkers have been searched. They are linked to different processes, such as amyloid production and deposition, neurofibrillary tangles formation, synapses loss or neuronal degeneration and, among them, it is worth to mention sAPPα[181], BACE1[182,183], $A\beta_{40}$ and other truncated forms[184], neuroserpin[185], transthyretin, cystatin C, p-tau231[186], oxysterols[187], cytokines, chemokines, glia-derive proteins and ApoE, whose levels in the CSF varies according to the genotype[26].

Even though some of them were more concentrated in AD patients and were useful to predict pathological deterioration, others were not very promising, given that their levels were unchanged in both the healthy and diseased cohort. Also, despite the fact that these substances are easily measurable using commercially available assays, these techniques are subjected to high variations among different laboratories, since there are no standardized guidelines for samples processing[188,189]. Moreover, although these molecules are highly concentrated in the CSF, this biological fluid is not devoid of disadvantages. Indeed, the lumbar puncture for its collection is very invasive and it is not suitable neither for the initial diagnosis nor for the follow-up screenings.

Thus, several studies focused on peripheral blood, which is inexpensive, easily accessible and contains cytokines as well as growth factors, which could represent good biomarker candidates[190]. In addition, it has been shown recently that lymphocytes and platelets share biochemical similarities with neurons and display the same anomalies found in psychiatric disorders, such as oxidative stress, mitochondrial dysfunction, cell cycle alterations, elevated apoptosis and impaired calcium homeostasis[191–203]. Moreover, expressing both APP and tau, they could represent a valid and abundant source to detect AD molecular changes[204].

Unfortunately, none of these biomarkers has shown high specificity yet, so that the combination of different diagnostic tools is still the best way to identify the pathology[205].

1.6 Pharmacological Therapy

AD is considered a syndrome rather than a disease and its multifactorial nature complicates its cure[206]. Indeed, there are no definitive pharmacological treatments available nowadays but both symptomatic and aetiology-based therapies have been tried.

➢ Acetylcholinesterase inhibitors

The election drugs in the first case are acetylcholinesterase inhibitors (AChEI), such as donepezil, rivastigmine and galantamine, which promote higher ACh levels in the brain by inhibiting its degrading enzyme acetylcholinesterase[207]. Thus, they exert their positive action by restoring cholinergic transmission, which is altered due to abnormal Aβ accumulation[208,209]. Moreover, they have also displayed the ability to regulate α-secretase activity as well as the inflammatory response[210]. Excluding tacrine, which has been shown to be hepatotoxic, these substances are well tolerated and they have dose-dependent adverse effects[211].

➢ Anticholinergic drugs

On the other hand, several studies have tried to treat early symptoms of dementia targeting both muscarinic and nicotinic receptors by means of anti-cholinergic drugs that seem to ameliorate cognitive impairment in prodromal AD subjects, even though they display several side effects, such as constipation or urinary retention[212]. Thus, a long therapy with these medications is not recommended, given that they have been shown to increase AD risk and that this outcome is much more pronounced in older adults, due to decreased acetylcholine synthesis and increased BBB permeability[212,213].

➢ NMDA antagonists

Another option is to use NMDA receptors antagonists like memantine, which was approved by Food and Drug Administration (FDA) for the treatment of moderate-to-severe and it is used off-label in mild-to-moderate AD[211]. This molecule acts by blocking over-stimulation of glutamate receptors and by attenuating tau phosphorylation and it can be administered alone or in combination with AChEI[214].

➢ Serotonin receptors antagonists

Lastly, some pharmacological trials have focused on serotonin receptors, which are present in some areas involved in learning and memory and, when inhibited, seem to improve cholinergic transmission[207,215].

➢ Aetiology-based treatments

Differently, aetiology-based treatments have focused on the amyloid-β peptide, which is still considered one of the main culprits of AD pathology. The first drugs were designed to modify the amyloidogenic pathway, inhibiting the over-activation of β- and γ-secretases[216], whereas other molecules were developed to induce the up-regulation of α-secretases like ADAM10 or MMP-9[217]. Unfortunately, some trials targeting BACE1 were unsuccessful because of problems in drug delivery and toxicity[218], while interfering with γ-secretase activity has shown severe side effects, since this process induces deficiencies in BDNF axonal trafficking and signalling[219] On the contrary, potentiating α-secretase cleavage has been demonstrated to prevent the formation of Aβ peptide. In addition, some pharmacological treatments have been developed to avoid amyloid plaques deposition, through Aβ monomers stabilization and have been tested in clinical trials, displaying important side effects[215,220]. Another option could be stimulating ApoE transcription by means of specific agonists, in order to increase Aβ clearance.

Also, other strategies have tried to inhibit tau hyper-phosphorylation and aggregation, without showing any clinical benefit[207] and molecules suited for other pathologies have been experimented as well. Among them, statins were thought to have a positive effect on dementia, due to their role in cholesterol reduction but they actually failed in preventing cognitive decline[221]. Differently, anti-oxidants and anti-inflammatory drugs have shown mixed results[222].

- **Immunization**

However, in recent years, a different treatment has been suggested and it consists in both active and passive immunization.

In the first case, aggregated $A\beta_{1-42}$, in combination with different adjuvants, has displayed the ability to reduce both plaques and cognitive deficits in vivo[80,223], so different vaccines have entered clinical trials but some of them seem to trigger damaging immune responses[224–226].

In the second approach, peripherally administered anti-Aβ antibodies efficiently prevented fibrils formation and ameliorated behaviour[227], through either Aβ aggregates disassembly or microglia activation[78,228]. Moreover, a peripheral sink effect has been theorized and it hypothesizes that amyloid deposition could be blocked by means of soluble Aβ sequestration, which seems responsible for the drawn out of the same molecule from the brain. However, these antibodies showed some disadvantages, such as difficulty in target selection and BBB penetration, expensive costs, need for repeated injections, immune system activation and haemorrhagic risk[229,230].

Furthermore, since its accumulation seems to be better correlated with cognitive impairment in AD than amyloid deposition, tau protein has started to be considered a good candidate for passive immunotherapy[71,231,232]. Indeed, different compounds have been developed to target its hyper-phosphorylated form and are currently in clinical trials[207,233].

Thus, the best strategies might be the ones targeting both Aβ and tau oligomers, which appear to be the most toxic species[234,235], without affecting the physiological function of these proteins and, for this purpose, antibodies specific for some conformations only have been designed[235,236].

1.7 Non-pharmacological Therapy

Even though pharmacological treatments are still the most common therapies to treat AD, several studies have focused on preventive drug-free strategies, which can be associated to either lifestyle or diet.

- **Life-style**

The first group include physical activity, mental challenges, energy restriction and socialization[237]. Indeed, exercise has been shown to stimulate neurotrophins release at both central and peripheral level[238,239] as well as free radicals reduction. Moreover, engaging mental tasks and activities could protect against cognitive decline, increasing neuronal density, which is responsible for brain plasticity[237].

- **Diet**

On the other hand, different food compounds have shown neuroprotective properties. Among them, folic acid blocks Aβ accumulation, whereas flavonoids inhibit acetylcholinesterase activity and glutamate release[240]. Also, resveratrol is an anti-aging molecule capable of ameliorating mitochondrial dysfunction[241,242] and probiotics have anti-inflammatory effects, exerted by their action on the microbiota-gut-brain axis[243].

Therefore, a definitive treatment for AD has not been discovered yet and both pharmacological and non-pharmacological therapies are aimed at ameliorating symptoms or preventing them. Given that this pathology starts long before the overt dementia stage, it is mandatory to improve techniques for an early diagnosis. Indeed, reversing cognitive decline becomes more difficult as AD progresses, since mental deterioration is too extended. Thus, medications should be administered in pre-clinical or mild clinical phases, in order to increase their efficacy and minimize brain impairment[244–246]. Moreover, changings in biomarkers levels during the pathology would be more helpful than their measure at the baseline only, since it would allow a better diagnosis as well as a prognosis hypothesis[188,247].

For these reasons, it is fundamental to continue searching not only for AD aetiology but also for a causative treatment, which could definitely halt or reverse a severe pathology that is quickly spreading worldwide and represents a plight of an ageing population.

1.8 BPSD

The term *Behavioural and Psychological Symptoms of Dementia* (BPSD) was introduced in 1994 by the International Psychogeriatric Association[248,249].

➢ Overview

It represents an *umbrella* concept to describe a wide variety of disturbances usually manifesting during the course of AD pathology. These symptoms include anxiety[250], agitation[251], apathy[252,253], depression[254,255], psychosis, aggression[256], euphoria, disinhibition[257], hallucination[254,257], elation, delusion[258,259], misidentification, dysphoria, irritability, wandering, aberrant motor behaviour, eating disorders[260] and sleep disturbances[318]. According to the Cache County study, they have a 97% prevalence in dementia[261] and almost all patients experience at least one of them during the course of the disease[262]. They can appear at any time[263] and usually they worsen from mild to severe AD[264,265], albeit some studies suggest that they may be more common in moderate pathological stages[266]. Moreover, they can be either episodic or persistent over long periods and they are thought to be predictive of poorer outcomes, such as faster cognitive deterioration and early death. Also, these symptoms negatively impact not only on the patient itself but also on its caregivers, which experience reduced quality of life[267], worse health[268], stress and depression[269]. Indeed, most of demented subjects are nursed by their relatives[262] but the more BPSD increase, the higher family burden becomes, making home-care impossible. Thus, the presence of these symptoms often leads to a premature institutionalization, increasing the healthcare costs[270,271].
It has been demonstrated that BPSD may accompany not only overt dementia but also MCI[272]. Indeed, 35-75% of these patients experience at least one symptom and the most common are depression, apathy and anxiety followed by either irritability or agitation[273]. All of them are associated to later conversion to AD and, therefore, they could represent a clinical indicator to identify prodromal disease[274].

Considering demented patients, there are differences among symptoms included in BPSD spectrum, according to the clinical tests used to measure them and also depending on AD pathological stage. Indeed, hyperactivity and apathy show high persistence and incidence, which are moderate for depression and anxiety and low for psychotic symptoms[275,276]. Moreover, all these disturbances have been associated to a significant decline in cognitive functions and

memory[266,277]. In some studies, they worsen in early and moderate dementia and become stable in the final stages[266]. Also, affective symptoms are usually seen in milder impairment and younger age at pathology onset, whereas psychosis is typical of severe conditions and older-age onset[278,279].

➢ Clusters and tests

In order to help their analysis, BPSD can be categorized either into *clusters* or *factors*. In the first case, patients are grouped based on their symptom profile, leading to non-overlapping clusters whereas, in the second one, the groups depend on the symptom itself, so that individuals may belong to more than one group[280,281]. The categories include affective symptoms (dysphoria and anxiety but also apathy), psychosis (delusions and hallucinations), hyperactivity (mainly irritability and aggression but also disinhibition and aberrant motor behaviour) and euphoria. The most problematic disturbance is apathy, since it is important for predicting poorer outcomes and is observed to be loading on any factor[253]. These clusters may slightly differ among the studies, according to the clinical instrument used to measure BPSD[280,282].

The main one is the NeuroPsychiatric Inventory (NPI), which allows the quantification of the frequency and severity of 12 different symptoms[283,284] and its score usually increases over time. This test is administered to the caregivers, together with the Caregiver Burden Inventory (CBI), in order to evaluate also the distress experienced during patients' assistance. However, similar results in BPSD assessment are obtained using other clinical scales[285], such as Behavioural Pathology in Alzheimer's Disease Scale[286], Neurobehavioral Rating Scale[287] and Behaviour Rating Scale for Dementia[288], suggesting that these disturbances are not specific to NPI. These general instruments take into account not only symptoms alone but also correlations among them but, sometimes, this approach could introduce errors. For this reason, individual-symptom studies might be useful, even though they may hide the interactions among all disturbances. The most employed scales to assess BPSD individually analyse depression (NeuroPsychiatric Inventory Depression subscale (NPI-D) and Geriatric Depression Scale (GDS)) and agitation/aggression (Cohen-Mansfield Agitation Inventory (CMAI) and Behavioural Pathology in Alzheimer's Disease (Behave-AD) aggressiveness sub-scale).

➢ Aetiology

Unfortunately, BPSD biological causes have not been clarified yet. Some genetic associations have been found for psychosis and hyperactivity but not for affective symptoms[289] and the frequency of ApoE have been linked to aggression and psychosis[290,291]. Nevertheless, no genes seem to have a strong influence on BPSD manifestations. Moreover, higher serum $A\beta_{40}/A\beta_{42}$ ratio has shown connections to early-onset depression, whereas psychosis has been associated to serum BDNF levels[292], greater intracellular accumulation of hyper-phosphorylated tau and its CSF levels[293,294]. In addition, plasma GABA has been positively correlated with depression and apathy scores on NPI[295], whereas higher $A\beta_{42}$ accumulation has been related to agitation and psychosis values[296,297]. Besides biological triggers, environmental factors such as overstimulation and surroundings together with physical factors like pain and dehydration are believed to influence BPSD appearance[298]. However, whether these disturbances reflect neuropathological changes in AD or represent patients' reaction to cognitive impairment has not been established to date. Thus, unrevealing the biological processes underlying these disabling symptoms is fundamental to find a cure.

➢ Treatment

Given the lack of knowledge about BPSD causes, a definite pharmacological treatment has not been identified yet. There are no compounds approved by FDA, thus all drugs are used off-label. The most employed symptomatic therapies include antidepressants, mood stabilizers, cognitive enhancers and antipsychotics[299]. Indeed, these molecules, together with neuroleptics, have been useful in managing aggression and agitation but a long-term treatment is characterized by an increase in adverse events[300]. On the contrary, antidepressants are efficient in treating BPSD and they are well-tolerated by demented elderly patients, although they have shown side effects as well[301,302].

Apart from these compounds, also cholinesterase inhibitors have been used to manage neuropsychiatric disturbances. Indeed, donepezil improves affective and psychotic symptoms but it has uncertain effects on hyperactivity and no influence on elation[303,304], whereas galantamine has been useful in reducing psychotic but not affective disorders[305]. Nevertheless, these molecules are associated to severe adverse effects and this is also the case for benzodiazepines[304,306–308].

Beyond drug-mediated approaches, non-pharmacological therapies exist and include behavioural and environmental interventions, such as aromatherapy, massage, acupuncture, cognitive and memory training, reminiscence therapy, light therapy, Snoezelen room therapy, physical activity and walking programmes[263,309,310]. Moreover, caregiver support is fundamental to ameliorate BPSD. Indeed, when family members are properly taught how to manage these symptoms, improvements in patients' condition have been shown[263,311–313]. Although not devoid of any risk[309], all these alternative approaches should be employed as a first-line treatment for neuropsychiatric disturbances[314] but they are seldom included in clinical care because standard guidelines are still missing, as well as specialized staff, instruments and training programmes for caregivers[315,316].

Given the unknown and complex aetiology of BPSD, a multidimensional assessment of patients' condition is essential, to better target their needs and tailor interventions[317]. One single treatment approach is unlikely, due to the variety of symptoms. Even though is not clear whether their treatment slows dementia decline[318], multiple therapies might be helpful in reducing these severe and disabling disturbances, in order to mitigate both patients' and families' stress load and to reduce healthcare system economic burden.

Chapter 2

Neuroinflammation

2.1 Brain-periphery communication

The word neuroinflammation describes a damaging condition of the nervous system, characterized by the activation of the immune response and considered very peculiar because of the structure of the CNS itself. Indeed, it is surrounded by mechanical and biological barriers to protect the brain from external insults, it has a limited regenerative capacity and any impairment could lead to a definitive loss of function. For these reasons, neuroinflammatory processes contribute to neurodegeneration, dementia and functional impairment[319] and their involvement in AD is suggested by the presence of inflammatory mediators in AD brains[320,321], which are responsible for behavioural and metabolic changes and by the protective effect of non-steroidal anti-inflammatory drugs (NSAIDs) against disease development[322]. Moreover, chronic conditions with a systemic inflammatory component are able to prime the brain[323,324], which is particularly sensitive to inflammation-driven damage in some areas[325]. Also, the mechanisms of neuroinflammation in these pathologies are similar to the ones observed in aging, strengthening the contribution of this process to AD development[326]. The goal of neuroinflammation in the presence of a CNS disease would be to limit the extent of the pathology and to clear tissue damage, promoting its repair and regeneration. Unfortunately, if uncontrolled, this useful process could acquire a damaging role and could lead to neuronal loss and dysfunction[327,328].

Since the brain has been considered an immune-privileged organ for a long time[329], the infiltration of immune cells in the CNS was thought to be possible just in the presence of a blood-brain barrier breach, which is always accompanied by the production of pro-inflammatory cytokines and glial activation[330]. However, many studies have demonstrated that immune cells have various non-inflammatory roles in the brain and that there is a close strictly controlled relationship between the CNS and the peripheral immune system[331,332]. Indeed, cytokines influence spreads to a central level through several routes and ultimately impact on neuronal development, plasticity and function[333,334]. The main way allowing these molecules to enter and affect the CNS is represented by circumventricular organs, which lack the blood-brain barrier but other paths exist, such as cytokines stimulation of endothelial cells to secrete immune factors into the brain, direct transportation of peripheral cytokines through the BBB by means of saturable carriers and activation of the vagal afferents by circulating cytokines[335].

The bi-directional communication between CNS and peripheral immune system is known as neuro-immune axis[336] and deeply involves the neuro-endocrine system. Indeed, the main mediators of these interactions are not only cytokines and chemokines but also neurotransmitters and hormones, which guarantee adequate reactions to endogenous and exogenous stimuli, in order to respond in an adaptive physiological manner. The various stressors cause the activation of the hypothalamic-pituitary-adrenal (HPA) axis and of the sympathetic nervous system (SNS), which leads to the release of glucocorticoids and catecholamines, respectively[337,338]. These molecules are necessary to transfer the stress information from the brain to peripheral organs, so that the body can react properly[336,339]. Cytokines in particular are able to stimulate the production of cortisol, whose levels are altered in depressed subjects[340–342]. The HPA axis activity is also potentiated by activated microglia through the release of IL-1β. Moreover, hormones influence microglial function by inhibiting phagocytosis, reducing ROS production and NOS release[343].

Thus, various molecules and mechanisms account for the interaction between CNS and peripheral immune system and they are different in physiological and pathological conditions. The fine-tuned control of this communication is strictly related to the final neuroprotective or neurotoxic outcome and, to better understand it, it is indispensable to analyse the cellular populations involved in these processes.

2.2 Cytokines

These molecules, being the main mediators of the immune system, are necessary to maintain neuronal integrity but they may also have harmful effects in some circumstances, depending on their concentration, on the cells that are activated and on the presence of other factors in the microenvironment[320,344]. This is especially true for pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ, whose levels can increase not only following an inflammatory stimulus but also because of stress[345], leading to damaged neurogenesis and impaired neuronal homeostasis[346,347]. Indeed, IL-1β regulates BDNF activity but also stimulates immune cells to produce other pro-inflammatory cytokines, activates microglia and favours neurodegeneration by enhancing tau hyper-phosphorylation[348,349]. In a similar way, IL-6 influences Long Term Potentiation and memory but mediates the synthesis of acute phase proteins too[344,348]. Likewise, TNF-α modulates the synaptic strength and preservation but, on the other hand, has a role in apoptosis,

neurodegenerative processes, excitotoxicity and decreased proliferation[350] and, similarly, IFN-γ enhances neurogenesis but might also reduce it[351]. Not only pro-inflammatory cytokines but also anti-inflammatory ones are very important to regulate microglial function. They include TGF-β, IL-4, IL-10 and IL-13[352–354], which showed the ability to modulate Aβ-induced production of pro-inflammatory mediators[355,356]. Moreover, these factors are essential to allow tissue remodelling after injury and especially IL-10 is very effective in protecting the environment from different insults[357–359].

Alterations in cytokines levels are detected during an infection and are responsible for a set of changes overall known as sickness behaviour[360,361]. This condition, depicted by a depressive-like mood, lethargic features and disinterest in exploring the surroundings, eating and drinking[362], underlines the important relationship between immunological mechanisms and behavioural disturbances and reinforces the involvement of the immune system in generating brain vulnerability to psychiatric disorders[363–365]. Indeed, elevated concentrations of IL-1β, IL-6, IL-12 and TNF-α have been found in various mental disorders such as autism and schizophrenia[366–369]. On the other hand, chronic psychosocial stress is able to influence and activate the immune function[336,370] and it is responsible for the up-regulation of pro-inflammatory mediators in peripheral leucocytes[339,371].

2.3 Microglia

Microglia are considered the resident macrophages of the CNS and represent approximately 10%-15% of all brain cells[372,373]. They derive from an erythromyeloid progenitor in the yolk sac, which differentiates during embryonic development thanks to the interaction between colony stimulating factor-1 receptor (CSF1R) and its ligands colony stimulating factor-1 (CSF1) and IL-34[374–377]. This primitive microglial cell divides and, by means of active blood circulation[374,378], colonizes the brain in a heterogeneous manner[374,378,379] even in the post-natal period, once BBB is closed, demonstrating that this population self-renews and is replenished by proliferating microglial precursors throughout adult life[374,378–380].

➢ Healthy microglia

Microglia were initially described using a dichotomous paradigm, distinguishing between a ramified static condition and an amoeboid inflamed one, even though it is now clear that this second activated state can be displayed also in the absence of neuroinflammation and this mainly happens during foetal development and early post-natal life[381,382]. In the first case, the physiological quiescent cells, also known as resting, possess ramified processes to constantly screen the microenvironment[383–385]. Indeed, they are endowed with immune functions such as debris removal, infections restriction and tissue reparation but, on the other hand, they are also very important to maintain brain homeostasis during development, adulthood and aging[386]. Speaking of this non-immune context, they are implicated in multiple functions like activity-dependent synaptic pruning and stripping, excitatory synapsis maturation, axons growth, neuronal differentiation, astrocytes proliferation, angiogenesis and apoptosis[387–390]. To carry these tasks out, these cells count on a unique transcriptional signature TGF-β-dependent[391] and release trophic factors like BDNF, NGF, IGF-1 and GDNF, neurotransmitters and neuromodulators[384,392], which influence astrocytes and neurons[393].

Moreover, microglia constitutively express pattern recognition receptors (PRRs) and Toll-like receptors (TLRs)[394–396], which bind pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively), such as bacterial molecules, neuronal ATP or aberrant endogenous proteins[397,398]. Once these cells come in contact with an activating stimulus, they retract their branches to acquire a condensed structure[399] and they rapidly move towards the lesion site[400,401], where they promote an inflammatory response, which depends from surface receptors for complement, cytokines, chemokines and major histocompatibility complex II (MHC-II)[402,403] and which is normally self-limited, in order to restore tissue homeostasis[400,404]. Given their elevated plasticity, microglial cells are able to change their phenotype and function according to external stimuli, which deeply affect their differentiation, switching them towards a neuroprotective or a neurotoxic phenotype[405,406]. Indeed, following activation, these cells either assume an M1 pro-inflammatory or an M2 anti-inflammatory phenotype. In the first case, LPS or IFN-γ usually represent the trigger, whereas, in the second one, the response is initiated by IL-4 or IL-13. The M1-microglia produce TNF-α, IL-1β, IL-6, NOS, superoxide, hydrogen peroxide and matrix metalloproteinases, which are helpful in host defence but can also damage neurons[407]. On the contrary, M2-microglia release IL-10 and arginase-1 that promote tissue remodelling and

repair[387,408]. Although these extreme phenotypes are useful to describe microglia activation, transcriptome-based studies have demonstrated that there are many other dynamic intermediate heterogeneous functional states, which reflect a continuous spectrum rather than discrete groups[407,409,410] and this huge diversity is finely controlled by cytokines, which are fundamental in both physiological and pathological conditions in the brain.

➢ Microglia and ageing

Ageing is associated to immune senescence, that is overall deterioration of the immune system[411], which leads to a decreased capacity of fighting infections in elderly[412,413]. Indeed, in these subjects, microglia repairing abilities become dysregulated and these cells acquire an irregular distribution and shorter cellular processes[414–416], converting to a basal chronic mild activated state characterized by a decreased responsiveness to anti-inflammatory signals like TGF-β and IL-4[417–419] and by the upregulated expression of many inflammatory markers such as MHC-II and CD86[420,421].

This sensitization, which accounts for impaired physiological roles as well as for exaggerated immune responses to stimuli, is normally called microglial priming[335,418,422,423] and it might represent either a compensatory mechanism for microglia declining function or their incapability to return to a basal state[380,424,425]. A further boost to these primed cells comes from the upregulation of pro-inflammatory factors together with a deficit in the anti-inflammatory ones, which is typical of aged brains[418,426] and establishes a chronic enhanced activation of the immune system. This condition has been linked to an increased risk of developing cognitive dysfunctions, brain disorders and neurodegenerative diseases[427] and, indeed, ageing is considered a risk factor for cognitive decline, even though cognitive decline is not always present in ageing[428,429].

➢ Microglia and AD

Due to homeostatic mechanisms failure, uncontrolled immune reactions have been observed in AD as well. Indeed, Aβ aggregates cause neuronal stress and hypersensitization of microglia[430], inducing their activation. This process is considered an early event in the pathology[405,431] and, if these activated cells are not able to return to a basal state, it is responsible for disease worsening[432,433] and represents one of the main hallmarks of persistent and damaging neuroinflammation[377,434,435]. This condition is usually accompanied by chronic stimulation of

perivascular macrophages and astrocytes proliferation, since Aβ is able to induce the inflammatory process not only by indirect microglial stimulation but also through the inflammasome activation and the recruitment of reactive astrocytes and monocytes[436–438]. Moreover, the amyloid peptide is considered a direct trigger for inflammation, because its fibrils favour IL-1β-positive microglia recruitment at deposition sites and these cells seem to be involved in Aβ phagocytosis[320,439–441]. In support of this evidence, it has been shown that a strong pro-inflammatory stimulus within the CNS reduces amyloid load by enhancing microglial phagocytic activity and release of neurotrophic factors[442], even though some studies have demonstrated that these cells might no longer be able to process and degrade Aβ[443,444]. Thus, their beneficial role in preventing plaques formation is not permanent, since they soon become dysfunctional because of the altered expression of pro-inflammatory mediators[430,445]. These are thought to contribute to AD, given that their excessive concentration is profoundly harmful to neurons and brain microenvironment. Indeed, higher levels of IL-1β, iNOS, IL-6, TNF-α, chemokines, adhesion molecules and acute phase proteins are usually associated to an M1-phenotype in central immune cells and result in their long-lasting activation, which produces ROS, RNS and other neurotoxic substances, generating a vicious cycle of neuroinflammation[446–451]. Thus, this process might have a neuroprotective or a cytotoxic role, depending on the context and its precise function in the disease has not been clearly clarified yet[452]. Recent data seem to indicate that the microglial M1 phenotype is typical of early pathology[453], whereas the M2a state is more frequent in the advanced one and yields to greater Aβ deposits, because its phagocytic activity is impaired[454].

Moreover, abnormal cytokines levels are believed to be involved in the genesis of non-cognitive symptoms in AD patients[455,456], in which higher CSF levels of IL-6 and IL-8 or IL-10 have been associated to increased or lower NPI scores, respectively[284,457,458], as well as to depression and anxiety[459–461]. The same molecules have been found to be elevated in the serum and in the CSF of the same subjects[462–465] and some polymorphisms in immune genes represent even a risk factor for the pathology[466–468]. One of these genetic variants is located in the triggering receptor expressed on myeloid cells 2 (TREM2) gene, which promotes the M2a activation state of microglia and has been linked to an increased risk of late-onset AD[469,470]. Other susceptibility alleles have been found in the CD33 gene, responsible for inhibiting directly microglial Aβ uptake and in the ApoE one, which is involved in the amyloid peptide clearance[469,471–473].

Even though Aβ represents a trigger for microglial cells, it has also been shown to prevent plaques formation in immunization trials[474] and LPS stimulation lowered amyloid deposition as well, stimulating microglia clearing properties[475]. On the contrary, these cells are also identified in neurofibrillary tangles and they are able to influence tau phosphorylation but, in this case, LPS peripheral injection exacerbates the pathology rather than ameliorating it[476,477]. Thus, even though both Aβ and tau elicit microglial activation[478,479], they stimulate different aspects of the neuroinflammatory response linked to these cells and sometimes it is difficult to identify if the immune response has a beneficial or a toxic role in the brain.

To date, PET exams demonstrate that AD patients have an increased number of activated microglial cells, which well correlates with memory impairment[431,480] but the tracer normally employed as activation marker does not reflect the whole functional spectrum of microglia[481,482]. For this reason, it is more informative to parallel PET results with cytokines measurement in the CSF[462], even though both methods are not very practical, because either they are expensive or risky. Moreover, they could be influenced by peripheral cells entering the brain and expressing CNS antigens[483]. Indeed, it is now clear that microglia, monocytes and macrophages share many surface markers and also that the central immune response parallels the one outside the CNS[484]. Thus, many concepts related to these peripheral cells have been also applied to neuroinflammation[355].

2.4 Peripheral Monocytes

Monocytes derive from a haematopoietic precursor located in the bone marrow, which is able to self-renew and gives rise to incompletely differentiated cells belonging to the phagocytes system[485,486]. Similarly to microglia, the main cytokine driving monocytes development is M-CSF, also known as CSF-1, which seems to stimulate their production by inhibiting their apoptotic death[487,488].

- **Healthy monocytes**

These cells are characterized by the expression of CD115, CD11c, CD14 and CD16 in humans or CD115, CD11b and Ly6C in mice[489,490], whereas the chemokines receptors CX3CR1 and CCR2 are

typical of both species. Moreover, in humans, depending on the levels of these markers, it is possible to identify different subsets called classical ($CD14^{++}$ $CD16^{-}$), intermediate ($CD14^{++}$ $CD16^{+}$) and non-classical ($CD14^{+}$ $CD16^{++}$)[489,491,492]. These subgroups become two in mice, since they possess a pro-inflammatory subpopulation ($CX3CR1^{low}$ $CCR2^{+}$ $Ly6C^{high}$) and an anti-inflammatory one ($CX3CR1^{high}$ $CCR2^{-}$ $Ly6C^{low}$)[493,494], which is responsible for patrolling the luminal endothelium thanks to the interaction between LFA-1 and ICAM-1[495–497]. However, even though this subdivision is useful to identify the main function of these cells, it is now clear that their composition, like the microglial one, is more complex and wide.

Monocytes represent phagocytes involved in the organism defence against external or internal stressors. For this reason, they are endowed with scavenger receptors, low-density lipoprotein receptors, TLRs, chemokines and cytokines receptors, allowing them to recognize PAMPs and DAMPs[490,498,499] and they also express MHC-II, which makes them able to present antigens[500,501]. Indeed, being circulating phagocytes, they monitor the environment to maintain homeostasis and, in physiological conditions, they are able to enter the CNS either through the blood-CSF barrier or across the subarachnoid space[502–504]. This transfer of immune cells with intact BBB has been recently confirmed by the discovery of a lymphatic system in the dural sinuses[332]. Once in the target tissue, monocytes differentiate in macrophages[505], which are important for phagocytic activity, host defence, removal of apoptotic cells, remodelling of extracellular matrix, tissue homeostasis and development[506,507]. Indeed, they are a source of growth factors to perform tissue-repair[508,509] and, just like microglia, they show the ability to polarize their phenotype towards a wide functional spectrum[510]. This is characterized by an M1-pro-inflammatory state, which is important as a defence mechanism against microbes or by different non-classical activation ones[511,512]. These are classified as M2a-wound healing (stimulated by IL-4 and IL-13, involved in extracellular matrix remodelling and characterized by high IL-1Ra and arginase expression), M2b (stimulated by immune complexes, TLR activation and IL-1R ligands, characterized by high arginase, IL-1β, TNF-α, IL-6 and low IL-12 expression and identified by the specific marker CD86) and regulatory-M2c (stimulated by IL-10, involved in anti-inflammatory functions and characterized by high TGF-β and IL-10 expression)[513,514]. This last state can also be induced by the glucocorticoids produced following HPA axis activation and it helps the survival of organisms[515].

Given their repairing properties, macrophages always try to restore homeostasis alone but, if inflammation is highly severe, they might need external help, so they recruit neutrophils and monocytes from the periphery.

➢ Monocytes and AD

Many evidences suggest that a systemic inflammatory response could strongly result in leucocytes infiltration from the blood to the parenchymal perivascular space of the CNS, leading to neuroinflammation and neurodegeneration[504]. Indeed, the initial trigger activates microglia, which release pro-inflammatory mediators favouring BBB permeabilization to monocytes[516,517]. Moreover, these migrating cells are sensitive to chemokines gradients and their recruitment into the brain is assisted by the upregulation of adhesion molecules on the endothelium, in order to guide their rolling and diapedesis[518–520].

In physiological circumstances, monocytes are restricted to CNS borders but, during aging and pathological conditions, monopoiesis is highly induced by GM-CSF upregulation[521] and BBB permeability increases[522], so that these peripheral cells can quickly and easily reach the brain when required, acquiring different phenotypes according to the entrance site[523] and expressing chemokines receptors that guide them towards the injured site[524–526]. Even though they share the same morphology, these infiltrating monocytes might be distinguished from resident microglia thanks to specific markers and they seem to have distinct roles in pathological conditions like AD[442,527]. Indeed, whether microglia prevent amyloid plaques formation, monocytes-derived macrophages clear Aβ deposits more efficiently[494,526,528]. This microglial cells inability to recognize damaging molecules most likely stems from their long-life exposure to CNS environment, which induces a tolerance state in them and makes the presence of additional phagocytes necessary[529,530]. Their brain infiltration is further supported by different studies demonstrating that the immune cells found around amyloid plaques do not belong only to the resident microglial pool but also to the myeloid progenitors one[441,531]. Indeed, preventing monocytes recruitment increased Aβ deposits, confirming their important clearance role[526]. However, despite their essential function, these cells have been shown to display poor differentiation and impaired phagocytosis both during aging and AD[532,533] and their number is decreased in MCI patients compared to AD ones[534].

There are different chemokines responsible for peripheral monocytes attraction to the CNS, such as CCL7 or CCL5 but the main one is CCL2, which is also known as monocyte chemoattractant protein (MCP-1)[528,535,536]. Following an inflammatory stimulus, this molecule is strongly upregulated and induces an increased differentiation of bone marrow precursors[537,538]. On the other hand, it seems to regulate BBB permeability to facilitate leucocytes transmigration and, indeed, it has been shown to contribute to barrier opening during their extravasation[539,540]. Also, its mRNA levels are altered in peripheral cells of AD patients and Aβ is able to induce its production, stimulating monocytes infiltration and differentiation in macrophages in a dose-dependent manner[541]. Moreover, it is highly produced by microglia surrounding amyloid plaques in the brain[542,543] and it is believed to intensify their formation by enhancing ApoE expression[544,545]. It also displays higher levels in the CSF, serum, plasma and PBMC of both AD patients and MCI-to-AD ones and this increase not only is considered an early event in the pathogenesis[546] but also seems to correlate with a faster cognitive decline[247,547]. However, it is not clear whether this molecule represents a risk or a protective factor, because in some circumstances it seems to boost microglial Aβ uptake[544]. Some studies have demonstrated that knocking-out the CCL2 receptor (CCR2), which is mainly expressed on the surface of monocytes[548,549], reduces both their migration to the CNS and anxiety-like behaviour, underlining CCR2 important role in peripheral cells infiltration and strengthening the influence of this process on the genesis of psychiatric disturbances[550–552].

In addition, peripheral immune challenges, as well as ageing, represent a second hit for already primed immune cells[455,553,554] and enhance their activation. In case of underlying AD pathology, these stimulations induce cognitive and behavioural impairments, together with further disease progression[555–558] but it is not clear yet whether they contribute to initiate AD or if they just accelerate it[427,559,560]. Also, these deficits are accompanied by increased levels of pro-inflammatory cytokines[561]. On the other hand, the chronic inflammatory condition responsible for microglial priming can cause BBB damages in early pathological stages[562,563]. This barrier is very important to eliminate the amyloid peptide in collaboration with the peripheral immune system, through different mechanisms like Aβ oligomer enzymatic degradation, Aβ transport by special carriers or cerebral interstitial fluid, Aβ phagocytosis by patrolling monocytes or microglia[526,564]. Moreover, perivascular macrophages, which represent a distinct cellular population replenished by circulating monocytes and expressing CD163 as well as CD206, act as antigen-presenting cells and actively

respond to brain inflammation, lowering amyloid deposits and helping BBB with Aβ clearance[374,377,565].

However, when the peptide accumulation in the perivascular space worsens, it is responsible for the decreased expression of tight junctions between endothelial cells and causes an increased BBB permeability. Due to this leakage, blood-born molecules highly concentrate in this area and trigger BBB total breakdown[563].

2.5 Neutrophils

Representing 50-70% of human circulating leukocytes, neutrophils are actively involved in acute inflammation and they quickly localize in the injured area to promote pathogens killing and clearance.

➢ Neutrophils in Physiology

In physiological conditions, these polymorphonucleates derive from bone marrow precursors that differentiate due to GM-CSF exposure[566] but the mature cells are also found in spleen, liver and lung[567]. They are very short-lived and, following cellular ageing, they up-regulate CXCR4 that guides them back to the bone marrow, in order to be eliminated. Moreover, they contain azurophilic granules filled with myeloperoxidase (MPO), secondary granules containing lactoferrin and gelatinase granules including MMP-9[568], which are all used to fight infections. However, given that the content of these organelles is highly damaging for the host itself, neutrophils' response to pathogens has to be strictly regulated. Normally, they are able to remove these inflammatory triggers by means of phagocytosis, degranulation or Neutrophil Extracellular Traps (NETs) release.

➢ Neutrophils Extracellular Traps

In case of infection, immune resident sentinels in the damaged area produce inflammatory mediators that induce changes on the endothelial surface, in order to facilitate neutrophils recruitment [569]. Following an IL-1β or a TNF-α chemotactic gradient, these cells start to roll inside blood vessels and contact the ICAMs proteins on the endothelium with their lymphocyte function-associated antigen (LFA-1). This bond might slow down neutrophils rolling [567,570], which is followed

by full arrest and crawling [571,572]. This process is necessary for these cells to transmigrate through both the endothelium and the basement membrane, involving several adhesion and junction proteins [573,574]. Once neutrophils reach the injured tissue, they orchestrate an immune response to eliminate the inflammatory trigger and NETs release represents a recently discovered mechanism used by these activated cells to trap and eliminate extracellular pathogens[575]. These assemblies consist in large, web-like structures containing cytosol and granules proteins such as neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, calprotectin, cathelicidins, defensins, which are all attached to decondensed chromatin[575] mainly deriving from nuclear DNA but also from the mitochondrial one[576]. Their release, which is called NETosis[577], starts with neutrophils' depolarization, inducing nuclear envelope disassembly and chromatin decondensation[577,578]. This process causes DNA to mix with granules proteins and it is often accompanied by modifications like histone deamination or citrullination[579,580]. Next, following plasma membrane permeabilization, the cellular content expands outside the cells, few hours later their activation and eliminates the inflammatory trigger. NETosis are normally stimulated by large pathogens that could not be ingested by phagocytosis and strictly depend on ROS production as well as NE nuclear translocation, in order to elicit chromatin decondensation[581,582]. Moreover, it has been demonstrated that NETs formation might be promoted by neutrophils interaction with platelets formerly stimulated by LPS[583].

➢ Neutrophils and AD

Several evidences have shown that circulating neutrophils infiltrate AD brains in different mouse models and accumulate near amyloid plaques in a non-random manner[584]. In line with that, oligomeric and fibrillary Aβ peptide seems to suppress the apoptosis of these cells[585] and to induce their chemotaxis through both LFA-1 transition to its high-affinity state and ICAM up-regulation[586], which facilitate cellular adhesion to vessels endothelium for subsequent extravasation[586]. It has been proposed that plaque-associated glial cells might release chemokines to attract neutrophils and that these cells migrate towards amyloid deposits to engulf them and to allow their further elimination by microglia[584,587]. However, even though their role in the pathology is still debated, there are evidences of IL-17 and NETs presence in AD mice brains[586], where they have been demonstrated to induce cognitive deficits and neuropathological changes[584,586]. These impairments appear to be rescued by neutrophils depletion as well as by the transient blockage of

LFA-1 integrin, which controls their vascular adhesion, extravasation and intraparenchymal motility[586] raising the possibility of a therapeutic treatment limiting their migration and infiltration[586,588,589]. Moreover, the animals in which cognitive functions were restored displayed less microgliosis, leading to the hypothesis of a neutrophils-dependent glial cells activation that establishes an inflammatory vicious cycle[590,591].

Similarly, neutrophils and NETs have been also identified in both blood vessels and brain parenchyma of human AD patients, indicating a possible damaging role for their transmigration[584,586]. Indeed, the toxic phenotype acquired by these peripheral cells following activation has already been shown to compromise BBB and neurons[592–594]. Moreover, NETs were observed near amyloid deposits in diseased subjects[595] and seem to degrade Aβ fibrils into oligomers that could, in turn, boost neutrophils stimulation. Also, upregulated levels of pro-inflammatory cytokines have been detected in brain as well as CSF of both AD patients and MCI ones, in comparison with healthy individuals and it has been proposed that they could contribute to intravascular NETosis[596]. On the other hand, some studies have demonstrated that AD is associated to an increased number of neutrophils, which also display a more elevated oxidative stress burden. Furthermore, in diseased subjects, a higher neutrophil/lymphocyte ratio (NLR) has been identified and it could be used as a marker to recognize peripheral inflammation in the pathology[597], even though this parameter displayed limitations in predicting MCI-to-AD transition[598].

For these reasons, neutrophils seem to be deeply involved in the inflammatory response accompanying neurodegeneration and their role in AD offers new possibilities as regards pharmacological therapy. Thus, they deserve a comprehensive study, in order to unravel their beneficial and detrimental functions in the pathological context.

➢ Neutrophils and Inflammasome

As previously said, NETs contain decondensed chromatin and it has been demonstrated that histones released in the extracellular space act as DAMPs, boosting the inflammatory response through the stimulation of TLRs and NLRP3 inflammasome[599], which was first described in 2002[600] and consists of an intracellular protein complex formed by nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing-3 (NLRP3) sensor, apoptosis-associated speck–caspase recruit domain (ASC) adaptor and pro-caspase-1[601]. Its activation is normally

triggered by several microbes or aggregated Aβ[602] and leads to caspase-1 production, followed by IL-1β cleavage[603]. However, this is a two-steps process involving a priming phase to transcribe NLRP3 as well as pro-IL-1β and a second stage characterized by the assembly of the whole complex. To date, amyloid peptide phagocytosis is believed to be the first stimulus in NLRP3 inflammasome activation[602], whereas CD36 represents a mediator to convey the signal from Aβ to NLRP3 protein[604] and, in accordance with these data, high levels of IL-1β and active caspase-1 have been found in brains of AD patients. On the contrary, the concentration of these proteins is unaltered in healthy subjects, suggesting that they could assemble the inflammasome complex but, differently from diseased individuals, they lack the indispensable signals to trigger its activation[605].

Interestingly, familial AD mouse models deficient in either NLRP3 or caspase-1 gene have shown improvements in cognitive decline, no spine loss in cortical neurons as well as reduced amyloid deposition[605], even though APP processing and expression were unaffected. Moreover, they displayed increased microglial Aβ phagocytosis, raising the possibility that inflammasome activation contributes to AD pathogenesis also through the impairment of this process[605].

Given that the abnormal activation of NLRP3 inflammasome has been linked to multiple impaired functions[606,607], this process should be strictly controlled and could represent a target for a pharmacological therapy aimed to ameliorating AD symptoms.

2.6 Delirium

Delirium is one of the most common acute-onset and severe neuropsychiatric syndromes affecting hospitalized elderly patients.

- **Overview**

According to the DSM-V, it is characterized by impaired consciousness, heterogeneity, fluctuation over time[608–610] and inattention, which is considered its hallmark feature[611,612]. These symptoms are often accompanied by other disturbances[609] either cognitive (memory deficits, disorientation or language difficulties), emotional or behavioural (sleep-wake disorders, affective lability, psychosis, delusions and motor problems)[613–615]. This syndrome is described as a confusional state

due to a dysfunctional homeostatic reaction following precipitating factors called *direct brain insults*, such as changes in medication, surgery, trauma, or metabolic imbalances[611,616,617]. However, there are also major predisposing elements to this condition. Indeed, individuals with more vulnerable brains because of advanced age or pre-existing dementia[613,618], can manifest delirium following mild noxious stimuli, which would be harmless to healthy people[619]. Linking these *aberrant stress responses* to the pathology is definitely more difficult because they consist mainly in the activation of the hypothalamic-pituitary-adrenal (HPA) axis and in peripheral or systemic infections[333], which don't involve directly the brain and make delirium under-diagnosed in 32-66% of patients[620–622].

➢ Clinical features

The overall prevalence of this pathology among bedridden patients is higher than 10% but it rises to 20% in older ones and exceeds 50% in post-operative hip fracture subjects[623]. Depending on the level of arousal, delirium can be hypoactive, hyperactive or mixed[624,625] and in all these variants there may be HPA axis activation. The mixed type is the most common[626] but shifts between hypoactive and hyperactive forms are also frequently observed[627]. This condition is usually a transient disorder, lasting hours to days but some patients experience longer episodes called *persistent delirium* and still exhibit symptoms months after the onset[628]. Regardless of its duration, this pathology is associated with longer periods of hospitalization, higher mortality, increased likelihood of dementia and acceleration of pre-existing cognitive decline[618,629–631]. Prior impairment in cognition, especially, is the most common risk factor involved in the development of *delirium superimposed on dementia* (DSD). Its prevalence in demented patients, ranging from 13% to 89%[618,632], is much more higher than that in cognitively unimpaired individuals and up to 76% of DSD subjects die within one year from the first episode[618,633]. However, identifying this particular condition is quite challenging, because confusion in older patients is often ascribed to ageing or dementia itself and is not considered as an acute disorder[634]. Despite the existence of screening tools to detect delirium like the Memorial Delirium Assessment Scale[635], the Delirium Rating Scale–Revised–98[636] and the Cognitive Test for Delirium[637], a deep knowledge of the baseline cognitive performance of the patient is necessary. Otherwise, the neuropsychological impairment measured by these tests could be attributed to mild or severe AD, also given that inattention is present in both early and advanced stages of the disease[638]. It has been proposed

that delirium may become a chronic disorder in demented subjects, manifesting with persistent symptoms interrupted by acute episodes[639,640] and it would be useful to develop objective assessment of attention and detailed evaluation of consciousness levels[641,642] to help distinguishing between the two conditions.

➢ Aetiology

As regards delirium causes, they have not been fully identified yet[624] but the two main hypothesis point to cholinergic deficiency and neuroinflammation.

The first theory argues that the CNS modulates the innate immune response by means of the so-called *cholinergic anti-inflammatory pathway*, that is the descendent part of the *anti-inflammatory reflex*, comprising afferent and efferent fibres of the vagus nerve[643]. Indeed, its activation leads to the suppression of LPS-induced systemic inflammation[643,644], through the peripheral release of acetylcholine. This neurotransmitter exerts its anti-inflammatory effect by binding to the α7 nicotinic receptor, which is expressed especially in the spleen but also in microglial[645] and immune cells[643,646]. This homeostatic mechanism is necessary to maintain the pro-inflammatory responses within an adequate range[643] and its efficacy depends on the expression of acetylcholine receptors in periphery[647]. Cholinergic signalling in the brain is important for arousal, sleep, attention and mnemonic processes[648]. In confirmation of this involvement, when inflammation interferes with ACh release, transient deficits of the working memory are detected[649,650]. Moreover, anticholinesterase drugs used for AD could be beneficial for delirium[651–653] which, on the contrary, may be induced by side effects of anticholinergic treatments[654,655]. Thus, an altered cholinergic function seems to have an important role in cognition disturbances and could also be considered as a risk marker for delirium itself[656]. Indeed, individuals developing this post-operative condition show lower plasma cholinesterase activity even before surgery whereas high serum anticholinergic activity is associated with a severe pathology[657].

The second theory, instead, claims that delirium is precipitated by systemic inflammation[658–660]. Indeed, this condition can induce acute changes in working memory in both aged and already-experiencing-neurodegeneration animals[661]. These alterations are comparable to those observed in elderly and demented patients according to DSM-IV and, usually, after these episodes, these subjects' cognitive deficits get worse.

The body response to an infection or surgical intervention is known as sickness behaviour[662] and it is characterized by the production and release of pro-inflammatory cytokines, activation of the inflammatory pathways and recruitment of immune cells. Moreover, it accounts for mood changes, fatigue, psychomotor slowing, lack of energies and motivation, anorexia, sleep disturbances, depression, anhedonia, anxiety and memory deficits[663]. Anti-inflammatory mediators are able to restore homeostasis but if they fail in counteracting this acute reaction, the pro-inflammatory response will be too strong and harmful to the tissue itself. An imbalance of this sensitive mechanism is believed to be responsible for delirium[664,665] but it might not be sufficient to induce this condition in healthy brains[666]. However, if a trigger like genetic predisposition, ageing or neurodegeneration is already present, even mild inflammatory stimuli can cause excessive and uncontrolled reactions leading to severe cognitive deficits[629,667]. Moreover, every successive challenge is more difficult to recover[668]. Thus, delirium can be considered a maladaptive response of the brain to systemic inflammation, due to a lower functional reserve and its seriousness depends also on the area affected by prior vulnerability[669].

Consistent with cytokines' pathogenic role, higher plasma levels of IL-6, IL-8, IL-10 and C-reactive protein have been found in delirium patients[611,670,671] whereas, comparing pre- and post-operative subjects, only IL-8 has shown increased concentrations in CSF[672]. Although these results have not always been confirmed[673], raised pro-inflammatory mediators such as TNF-α and IL-6 have been associated to more severe neuropsychiatric manifestations and faster cognitive decline in AD patients[460,670,674], since they are able to boost ROS production and neuronal cell death[675]. These outcomes are supported by different animal studies, in which systemic administration of LPS induces a robust immune response, responsible for behavioural anomalies similar to those experienced by delirious subjects[667]. Beyond evaluating the pro-inflammatory reaction per se, it could be useful to take into account both pro- and anti-inflammatory mediators' levels in different body fluids, to monitor the homeostatic breakdown in its entirety[676]. Moreover, HPA axis' dysfunction is often observed in elderly and demented patients and it is responsible for immune changes and cognitive deterioration[677–680]. Indeed, in delirious subjects and also in healthy ones after systemic injection of LPS or IL-1β[681], elevated plasma and CSF cortisol levels have been found. Moreover, high concentrations of this substance may be responsible for delirium precipitation in vulnerable subjects[682,683] and restoring their corticosteroids' baseline requires longer time[684,685]. On the other hand, HPA axis activation leads to glucocorticoids' production and these molecules can contribute to microglial priming in some cases, even though they have an anti-inflammatory

function[686,687]. Indeed, delirium patients show not only microglia but also astrocytes activation[670,688].

➢ Therapy

Given the multifactorial aetiology of delirium, there are no safe and effective licensed treatments available to date. Pharmacological approaches are aimed at reducing symptoms using antipsychotics or sedatives[689] but these medicaments have severe adverse effects[690], are not always effective[691] and, in some cases, may aggravate confusion and prolong its duration[692]. Thus, delirium prevention is achieved using other strategies, including early ambulation, frequent orientation to surroundings, consistent use of hearing aids and glasses, cognitive stimulation, optimal nutrition and hydration[693]. Indeed, the incidence of this pathology has been demonstrated to be higher in hospitalized subjects than in home-treated ones[694,695]. Even though their efficacy is not always evident, non-pharmacological treatments are preferred as first-line intervention to avoid a complicated condition, which is very stressful not only for the patient itself but also for the caregiver[613,614].

Although delirium represents a huge medical problem, there is no widely accepted animal model to study this condition to date. This is due to its multifactorial aetiology and also to its clinical characteristics. Indeed, being inattention its main hallmark, prefrontal cortex abnormalities are clearly involved in its pathogenesis[696,697]. However, most of research considering inflammation and cognitive disturbances concentrates on hippocampus only, which is not the area responsible for pathological symptoms. Thus, to achieve significant results, it could be useful to change the experimental paradigm, in order to focus on brain areas really implicated in this condition.

Chapter 3

TSPO/DBI system

3.1 TSPO Overview

In contrast to Central Benzodiazepine Receptor (CBR), which is expressed in the CNS only[698,699], Translocator Protein 18-kDa (TSPO) is a peripheral site for benzodiazepines' (BDZ) binding[700,701]. Indeed, after being first described in 1977, it was called Peripheral Benzodiazepine Receptor (PBR), because of its ability to form a high affinity complex with diazepam[701]. Although its mRNA is ubiquitously present in the whole body, PBR expression is not homogeneous among and within different tissues[701], probably because of a distinct translational regulation[702]. The highest levels are detected in endocrine tissues such as adrenal glands and gonads[701], whereas intermediate levels are typical of kidney and heart. Instead, PBR is present in very low levels in liver and healthy brain[701,703], in which it is mainly found in ependymal cells, choroid plexus[704,705], some neuronal populations[701,706], microglia[707,708] and reactive astrocytes[709]. Moreover, it is also expressed in platelets as well as in polymorphonucleates[710] and peripheral blood mononuclear cells, especially monocytes[711,712].

As concerns subcellular localization, this receptor is mainly placed on the outer mitochondrial membrane[701] but it has been found also on the plasma membrane of some cells like erythrocytes and monocytes[713,714], in the nucleus[714–716] and in microsomal compartments such as Golgi apparatus, lysosomes and peroxisomes[715].

➢ Structure

TSPO is an integral membrane protein that consists of 169 aminoacids forming a five α-helices structure, with an extra-mitochondrial C-terminal and an intra-mitochondrial N-terminal[717–719]. It crosses the lipid bilayer because of its high hydrophobic properties and tryptophan abundance[718,720]. Moreover, the C-terminal contains a CRAC motif, which is able to recognize and bind a cholesterol molecule with high affinity[721], providing that the site is not mutated in specific positions[722].

➢ Gene

This protein is encoded by a highly conserved single-copy gene located on chromosome 22q13.3 and containing 4 exons and 3 introns[723]. It is an ancient but non-essential gene[724] and this

characteristic makes it more suitable to be involved in disease pathogenesis[725]. Moreover, exon 2 contains a single-nucleotide polymorphism (SNP) classified rs6971, which causes a non-conservative alanine-to-threonine substitution in position 147. Being it near the CRAC motif[717,721], it has been suggested that it could affect cholesterol binding[726,727]. Indeed, the threonine allelic variant has been associated with reduced pregnenolone production by immune cells in both heterozygous and homozygous individuals[728], suggesting its dominant effect in altering neurosteroids' production. Given the role of these molecules in mood control[706], the SNP has shown a higher frequency in patients suffering from depression and adult separation anxiety[728]. Moreover, it seems to increase susceptibility to panic disorder[729] and to be associated with bipolar disorder diagnosis[730]. Thus, this mutation seems to be linked to different psychiatric conditions and deserves further detailed studies.

3.2 TSPO in Physiology

Even though CBR and PBR share a common ligand, they have different pharmacological, structural and functional properties.

Indeed, the first one forms a complex with central $GABA_A$ receptor, which is responsible for inhibitory neurotransmission whereas the second one is an 18 kDa protein, ubiquitously expressed throughout the body[731], whose function has not been clearly elucidated yet. Given its channel-like structure[732] and its high affinity for cholesterol, PBR was thought to act as a carrier to move this molecule from cytoplasm into the mitochondria[733]. Thus, its name was changed into TSPO to underline its transporter role, which seemed to establish a link between cholesterol mitochondrial import and steroids' biosynthesis[701,706].

➢ Functions

Indeed, this receptor is considered to have an important role in neurosteroidogenesis and neurosteroids' biosynthesis, of which it controls the rate-limiting step[734]. This hypothesis was supported not only by TSPO interaction with steroidogenic acute regulatory protein (StAR), necessary for cholesterol binding to TSPO itself[735] but also by several animal experiments showing that functional inactivation of this protein causes an early embryonic lethal phenotype[732].

However, this findings have recently been confuted by both in vitro and in vivo techniques, claiming that TSPO is not indispensable for steroidogenesis and might not even be involved in cholesterol transport[724]. However, some evidences still support its involvement in the process. Indeed, TSPO is a functional monomer but it also forms oligomers with itself or other proteins[736]. Its oligomeric shift could be induced by the dimerization of cholesterol molecules, given that their binding domain points toward the lipid bilayer[717] and could be important for the transporter functions of this receptor.

Beyond this controversial but most characterized role, TSPO has been implicated in many other cellular processes, such as regulation of cellular proliferation[737], respiration[731] and mitochondrial swelling[738], calcium signalling[739], immunomodulation[740,741], porphyrin transport and heme biosynthesis[742], anion transport[743] and apoptosis[744]. As concerns this last case, TSPO has been shown to regulate cytochrome C release from mitochondria and caspase activation[745], in response to cellular death signals such as oxidative stress, growth factor removal or exposure to cytokines like TNF-α[746]. Indeed, the receptor is considered one of the main components of the mitochondrial permeability transition pore (MPTP), together with voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and other proteins[732]. Its over-expression has been shown to affect mitochondrial dynamics and ROS production as well as pore opening[747] but recent studies have questioned its role, which is still under debate, demonstrating that this protein might not be necessary for the process[748].

TSPO also acts as an oxygen sensor[749], having protective effects from ROS damage and its low expression levels in neurons could explain the vulnerability of these cells. Moreover, it has been related to membrane biogenesis[750] and lipid metabolism, because of its interaction with ACBP, which is involved in fatty acids processing[751].

➢ Endogenous ligands

Given its multiple putative roles, TSPO has several different endogenous ligands in addition to cholesterol and porphyrins[701,732]. The main one is an 11-kDa polypeptide known as diazepam-binding inhibitor (DBI) because of its ability to inhibit diazepam binding to its $GABA_A$ and peripheral receptors[752], of which it is a negative allosteric modulator[753]. It is also called acyl-CoA binding protein and it is encoded by a single gene widely expressed in the CNS but mostly in glial cells[754]. The proteolytic cleavage of this molecule gives rise to more selective peptides named

endozepines, such as triakontatetraneuropeptide and octadecaneuropeptide, which stimulate steroids' biosynthesis[755]. Their local production is upregulated following increased TSPO expression[756] or Aβ stimulation[757]. Indeed, high levels of these peptides have been found the CSF of AD patients[758]. It has been demonstrated that all these endogenous TSPO ligands in vitro are able to stimulate chemotaxis[759,760], enhance the oxidative burst[761] and inflammatory cytokines' production[762], whereas they have the opposite effect in vivo[741].

3.3 TSPO in Pathology

Despite TSPO low levels in the brain, various physiological and pathological circumstances can modulate its expression.

- **Functions**

Indeed, its density is highly increased in activated microglia[763] and astrocytes[703], infiltrating macrophages and some neurons[701,703,764], following hormonal fluctuations especially involving the hypothalamic-pituitary-adrenal axis[765], ageing, diseased states such as obesity or cancer, acute and chronic neurodegenerative conditions like AD[734,766] or peripheral injury[706]. Thus, it can be considered a stress-responsive protein, since its levels rise as a consequence of different damaging conditions[708] and according to lesion's severity.

- **Exogenous ligands**

This peculiarity makes TSPO a good marker of reactive gliosis and neuroinflammation in different neurodegenerative conditions and also a helpful tool to identify accurately the injured brain area[706,767]. Indeed, high-affinity compounds were developed in the 80s[768], to allow in vivo visualization of activated microglia[769] and infiltrating macrophages in the CNS[770], using positron emission tomography (PET) or single-photon emission computed tomography (SPECT) exams[767,771]. The first prototypical synthetic molecule to detect TSPO is a non-benzodiazepine isoquinoline carboxamide known as PK11195[768], which is able to penetrate intact blood-brain barrier[772] and to bind selectively to its target[773]. The second classical ligand, instead, is the benzodiazepine Ro5-4864[774], which requires other components than TSPO to perform its functions. Both compounds

don't recognize $GABA_A$ receptors[768,775] and they have distinct thermodynamic properties as well as different binding sites on TSPO[717,776]. Indeed, PK11195 and Ro5-4864 are considered an antagonist and an agonist of this receptor, respectively[777,778] and their binding is up-regulated together with TSPO levels in the damaged CNS[779]. Despite their opposite action on the same receptor, they both regulate MPTP opening, inducing or impeding apoptosis according to the cellular microenvironment and alter mitochondrial respiration[780]. Moreover, they can stimulate neurosteroids' production by microglia and astrocytes, increasing cholesterol translocation into the mitochondria[701,706,734], although some results regarding this pharmacological ability are conflicting[781]. It has also been demonstrated that they might be therapeutic in neurodegenerative conditions such as AD, because they have a neuroprotective effect that depends on their ability to increase cell survival and neurosteroids' concentration[782]. Indeed, they are beneficial in limiting neuroinflammation[706], reducing microglia, macrophages and astrocytes activation, ROS and pro-inflammatory cytokines production[783]. Moreover, they regulate the viability and function of immune cells[784], boosting especially monocytes chemotaxis[760]. The biggest problem linked to the usage of classical tracers such as PK11195 and Ro5-4864 is the presence of a high number of binding sites, which are present on platelets, monocytes, erythrocytes and plasma proteins[785] and results in a low free fraction of the ligands, impeding their accurate measurement. For this reason, many other second-generation molecules have been developed, in order to increase the signal-to-noise ratio[767]. These ligands have an increased brain permeability and include imidazopyridines, benzothiazepines, benzoxazepines, indoleacetamide and phenoxyphenylacetamide derivatives, pyrazolopyrimidines and vinca alkaloids[700,706]. Despite their better imaging capabilities compared to PK11195 and Ro5-4864, these compounds have displayed differential binding depending on the TSPO genetic polymorphism rs6971[785,786], which is considered part of the binding pocket[717]. Indeed, because of its presence, the European population can be classified in 49% of alanine homozygous-high (HAB), 42% of heterozygous-medium (MAB) and 9% of threonine homozygous-low (LAB) binders[786,787] and these differences should be taken into account when performing PET exams, in order to avoid incorrect results, reduce sample size and increase statistical power. However, despite the numerous first or second-generation tracers, none of them has shown clear practical advantages compared to the others yet.

In addition to all these ligands, TSPO is also a target for benzodiazepines, which modulate circulating steroids levels[734,788] and bind also to the CBR in the CNS[701]. Because of their anxiolytic, anticonvulsant, antispasmodic and sedative-hypnotic action, exerted enhancing the GABAergic

transmission[789,790], they are widely employed to treat anxiety, insomnia and depression, even though they induce tolerance[790]. Taking the cue from benzodiazepines' role in these pathologies, other synthetic TSPO-binding compounds have been successfully used to treat anxiety[791] and to reduce neurologic and psychiatric diseases[706,791], underlining TSPO potential involvement in mood control. In support of this hypothesis, altered PK11195 binding has been found on platelets and lymphocytes of patients suffering from anxiety disorders[792]. On the other hand, Ro5-4864 has shown anxiogenic properties[793]. Moreover, TSPO expression is reduced in PBMC of subjects diagnosed with anxiety[794,795], generalized anxiety[792], social anxiety[796], adult separation anxiety[797], post-traumatic stress disorder[798] and panic disorder[797]. On the contrary, increased TSPO densities have been shown in major depressed[799] and in schizophrenic patients[367,800], even though these results have not always been confirmed[801]. Higher levels of this receptor have been found also in degenerated brains[802] and alterations in the number of binding sites are typical of stressful conditions[803].

Thus, TSPO appears to be an important element to produce neurosteroids, which are very important in physiological and pathological conditions but represents also a useful tool to both diagnose CNS damage and treat some psychiatric disorders. For this reason, further research regarding this receptor, its properties and functions is required, especially in light of the recent controversial results questioning its role throughout the body.

3.4 Neurosteroids

Circulating steroids are mainly produced by gonads, adrenal glands and other endocrine tissues but the term *neurosteroids* is only referred to the ones synthetized into the nervous system.

➢ Overview

This synthesis starts during development and remains into adulthood, in a region-specific manner depending on neurosteroidogenic enzymes and TSPO expression[804]. Indeed, this receptor controls the rate-limiting step of the synthesis process[805], represented by cholesterol import from cytoplasm into mitochondria[734,806,807]. Since the inner-mitochondrial membrane (IMM) is poor of this hydrophobic molecule, the protein StAR delivers it to TSPO[808], which is necessary to transfer it

from the OMM to the IMM, where the cytochrome P450 cholesterol-side-chain-cleavage enzyme (P450scc or CYP11A1) metabolizes it to generate pregnenolone (PREG)[734,809]. After processing, this precursor reaches the endoplasmic reticulum[734], to give rise to all other neurosteroids[701,734,766], classified in 3β-hydroxysteroids (pregnenolone sulphate (PS), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEA-S)), pregnane steroids (progesterone (PROG), dihydroprogesterone (DHP), allopregnanolone (3a,5a-THP), deoxycorticosterone (DOC), dihydrodeoxycorticosterone (DHDOC) and tetrahydrodeoxycorticosterone (3a,5a-THDOC)) and androstanes (androstanol and androsterone)[810].

- **Functions**

These molecules have different physiological neuroprotective effects such as inducing specific gene expression, sustaining the development and establishment of neural circuitry, ameliorating neuroinflammation, improving neuro-regeneration and controlling neurotransmission[811,812]. In particular, they are neuroactive endogenous positive or negative modulators of the GABAergic transmission[813–816] and some of them, such as DHEA, also enhance NMDA receptor activity. Thus, being able to activate $GABA_A$ receptor signalling, most of neurosteroids have anxiolytic and analgesic properties[816], even though they occupy a different binding site from BDZ[815,816] and, for this reason, they have an important role in neuropsychiatric conditions[817,818]. Indeed, alterations of their levels have been associated to different pathologies involving cognitive function and behaviour[706]. For example, lower levels of DHEA have been found in patients suffering from major depressive disorder[819], whereas a decrease in CSF concentrations of pregnane steroids such as allopregnanolone is involved in post-traumatic stress disorder (PTSD)[820]. Allopregnanolone is also reduced following prolonged isolation[821] or stressors exposure[822] and this reduction is responsible for behavioural anxiety, depression, schizophrenia and impulsive aggression[823]. In support of these evidences, TSPO ligands have shown in vitro and in vivo neuroprotective properties, consisting in the activation of neurosteroids production, which increases their levels in the brain and peripherally[782,791,824,825]. Higher concentrations of these molecules appear beneficial not only to brain cells but also to the patient's mood, especially because neurosteroids stimulate neurotrophins production.

Chapter 4

Neurotrophins

4.1 Neurotrophins Overview

Neurotrophic factors are endogenous secreted proteins important for proliferation, differentiation, developmental survival, neuroplasticity, pruning and myelination throughout life[826,827]. Among them, neurotrophins are the most powerful and best characterized. The first one was called Nerve-Growth Factor (NGF) and was described in 1949 by Hamburger and Levi-Montalcini, which formulated the *neurotrophic factor hypothesis*[828–830]. It states that developing PNS and CNS neurons need to retrogradely transport NGF into *signalling endosomes* through the innervating axons, from its production sites in target organs to the neuronal soma, in order to survive[828,829]. Nowadays, this endogenous compound belongs to the *classical neurotrophins* family together with Brain-Derived Neurotrophic Factor (BDNF), neurotrophine-3 (NT-3), neurotrophine-4/5 (NT-4/5) and neurotrophine-6 (NT-6)[831–841].

Beyond their main role in supporting neuronal survival[826], all these molecules have other functions such as modulation of network construction, neuronal migration and refinement of local connections[826,842], thus being indispensable mediators of axonal and dendritic growth and remodelling not only during the development but also in adulthood. Moreover, they are needed for receptor trafficking, neurotransmitter release, synapse formation and synaptic function[843] but they can also promote cellular death, depending on their biological form, the circumstances, the receptor and the pathway activated[844]. In some cases, they contradict the *neurotrophic factor hypothesis* because they can be transported anterogradely to deliver signals to post-synaptic cells[845].

Neurotrophins are synthesized in the rough endoplasmic reticulum and stored into secretory vesicles. They signal either through p75 neurotrophin receptor (p75NTR)[846,847] or tropomyosin-related kinase (Trk) receptor tyrosin kinases[848]. Although NGF, BDNF, NT3 and NT4/5 have the same affinity for the p75NTR, they show preferential binding for the Trk family members[848]. To be activated, they require a dimerization process for tyrosine trans-phosphorylation[849] and this could lead to the formation of Trk homo-dimers or Trk-p75NTR hetero-dimers[846]. Both receptors are expressed in specific areas within the nervous system and their levels are tightly regulated by dephosphorylation and degradation.

4.2 Nerve Growth Factor

Nerve Growth Factor (NGF) is a highly conserved glycoprotein[850] consisting of three subunits with different tasks: inactive α-NGF, biologically active β-NGF and processing γ-NGF. A single gene on chromosome 1 encodes the pro-NGF precursor[851,852], which undergoes a plasmin-mediated proteolytic cleavage of its signal peptide to produce mature 13-kDa NGF[853]. For a long time, pro-NGF was thought to be cut intracellularly and released as mature NGF in the extracellular space, in an activity-dependent manner[854,855]. On the contrary, it has been demonstrated that pro-NGF is secreted together with proteases and zymogens, which cleave it outside the cell and regulate the degradation of its very transient mature form, making the immature one predominant in human CNS[856].

It has important roles in neuronal survival, growth and maintenance[857] and it exerts these functions following p75NTR or TrkA receptor binding[858]. However, it can also induce apoptosis through p75NTR-sortilin activation[859]. Whichever receptor it binds, it needs non-covalent homo-dimerization to activate its pathways[860]. Its expression varies between developmental and adult stage[861] and it has been observed in neurons, glial cells, astrocytes and oligodendrocytes[862]. In the periphery, it has been found on platelets, muscle cells, Schwann cells and macrophages[863]. Its constitutive synthesis correlates with innervation density and influences axonal terminal sprouting, dendritic arborisation, neuropeptides and neurotransmitters production[864].

4.3 Brain-Derived Neurotrophic Factor

Brain-Derived Neurotrophic Factor has a complex genomic structure and possesses elaborated mechanisms to regulate its transcription, translation and post-translational modifications[865]. BDNF gene is located on chromosome 11 and gives rise to many alternative transcripts with different distributions and functions, according to the specific signalling pathway stimulated[866]. BDNF protein is synthesized as pro-isoform and cleaved to produce the mature form[867], the levels of which are affected by nutrition, metabolism, behaviour and stress[868].

➢ BDNF in the central nervous system

BDNF is widely expressed throughout the CNS[869] by neurons, microglia, astrocytes and vascular endothelial cells. Its expression and secretion are activity-dependent and very abundant in brain areas requiring high plasticity[870]. Indeed, BDNF is important for regulating synaptic structure, axonal sprouting, dendritic proliferation and adult neurogenesis[871], which are essential for spatial learning, memory and mood control[872]. All these trophic effects are activated following mature BDNF dimers binding to TrkB receptors[873], whereas the interaction between pro-BDNF and p75NTR stimulates the apoptotic pathway in peripheral neurons and glia[874,875]. Given its capabilities, this neurotrophin helps protecting neuronal cells from damage and its expression is altered by various brain insults[876,877]. Indeed, its chronic deficiency causes learning deficits[878] and its receptors are decreased in the pituitary gland during ageing, providing an explanation for the endocrine alterations seen in elder people[879]. Moreover, its gene contains a Val66Met (rs6265) polymorphism, which influences the trafficking and secretion of BDNF[880], possibly impacting on memory performances[881], even though some recent results concluded that this SNP is not associated to protein peripheral levels[882].

➢ BDNF in periphery

Even though the brain is the main organ producing BDNF, other non-neuronal tissues have also been proposed as its sources[883]. Among them, murine skeletal muscle has been largely considered, because it has been shown to express BDNF mRNA, whose levels increase following histone deacetylases inhibition[884]. This process is controlled by physical exercise, thus raising the possibility of a muscular-dependent BDNF release[885]. Although some studies indicated that higher concentrations of this neurotrophin derive from neurons within the skeletal muscle beds[886,887], it has been demonstrated that this protein is also produced by the very own contracting skeletal muscle cells, where it seems to enhance fat oxidation in an autocrine or paracrine manner[888]. Thus, it is rather unlikely that this muscular-derived BDNF is released in the circulation and operates in a hormone-like fashion[888]. Nevertheless, BDNF is considered a myokine, which is a word describing several molecules originating from the muscle fibres and acting in an endocrine or local way[889], in order to regulate intramuscular neurons survival, growth and maintenance[890] as well as skeletal muscle metabolism. Moreover, being a component of the hypothalamic pathway, it is also important to control body mass and energy homeostasis[891]. According to different animal

studies, physical exercise has been shown to exert a neuroprotective and neuro-regenerative role on hippocampus and cognition[892,893], probably through the up-regulation of peripheral BDNF levels[894]. However, these evidences are less consistent in humans, either they are elderly or MCI patients[895].

Further evidences supporting the link between the skeletal muscle and BDNF come from ALS research field and, in particular, from the myocyte enhancer factor 2 (MEF2) family of transcription factors, which includes four different isoforms (MEF2A-D) that are highly expressed in the brain as well as in myocytes[896]. In the first area, they are important for neuronal survival and synapses growth[897], whereas in the second one they guide skeletal, cardiac and smooth muscles development[898]. In addition, MEF2C and MEF2D expression has been detected in both lymphocytes and macrophages, having the role of boosting their proliferation and immune response, which are critical functions for modulating ALS pathology[899,900]. Moreover, these isoforms are directly involved in BDNF control, since they induce the production of distinct transcripts of its gene[901] and this interaction is further corroborated by recent data showing that BDNF down-regulation in PBMC of sporadic and $SOD1^{+}$ ALS patients might be connected to a MEF2C/MEF2D dysregulated activity[902].

On the other hand, another pathway has recently been highlighted in animal studies and it involves the myokine irisin, which is formed by the proteolytic cleavage of the transmembrane fibronectin type III domain-containing protein 5 (FNDC5)[903]. Through the circulation, the fragment reaches the adipose tissue, where it activates thermogenesis and exerts positive effects on adipocytes[904]. However, it could also cross the BBB to enter the CNS and, in this case, it stimulates BDNF production and hippocampal cells proliferation[905,906]. Irisin expression in the brain, as well as in the skeletal muscle, increases following sustained physical activity and decreases in connection with a sedentary lifestyle[907], indeed it has been called *exercise hormone*[903]. Several human studies have reported correlations between irisin concentration in the serum of healthy adults and their cognitive performances[908], whereas others have underlined positive associations of irisin levels with serum BDNF[909]. Thus, this myokine might represent the missing link connecting physical exercise to cognition and BDNF could embody the mediator of its effects[907,908]. However, since there are evidences of unchanged irisin levels after prolonged training, it might be possible that this molecule rises transiently after acute bouts of exercise and then returns to the basal condition[910,911].

Therefore, given its multiple inducing pathways, BDNF not only plays a fundamental role in the central metabolism but it also seems to have an important function in the periphery.

4.4 Neurotrophins and Inflammation

These neurotrophins are deeply influenced by neuroinflammatory conditions, which impair neuronal survival, proliferation and integration in brain circuits[912], thus ultimately influencing cognition[913,914]. Indeed, in vivo administration of pro-inflammatory cytokines or LPS mainly reduces BDNF gene expression but also decreases NGF and NT-3 levels[915] and it has been demonstrated that peripheral immune challenges can affect specific isoforms of BDNF[916], although how these inflammatory stimuli affect this molecule is not clear to date. Since a glucocorticoids-dependent reduction in neurotrophins levels has been observed[917], the hypothalamus-pituitary (HPA) axis, which is highly stimulated by pro-inflammatory cytokines[918], might be involved. Anyway, these mediators act also on glutamate and GABA[919], which are both able to modulate BDNF. Thus, the neuroinflammatory effect on this trophic molecule could depend on multiple mechanisms.

However, not only BDNF but also NGF is affected by inflammation. Indeed, its levels are up-regulated at the damaged site, due to the action of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6[920,921], which stimulate its production in neurons, Schwann cells, astrocytes, microglia, endothelial cells and other cell types[922–924]. Moreover, immune cells enhance their basal NGF production upon activation[925], together with TrkA receptors as well[926–929], demonstrating that this neurotrophin might affect the immune response. Indeed, it seems to act directly on monocytes migration, neutrophils and macrophages infiltration and it is believed to keep them in a *state of alert*, so that they can answer faster to danger signals[930]. On the other hand, it is essential for the activation of anti-inflammatory pathways that counteract an excessive inflammation[931]. This effect is mediated by both a direct and an indirect action on immune cells and might explain NGF increase as an attempt to restore homeostasis.

4.5 Neurotrophins and Ageing

Neurotrophins are affected not only by neuroinflammation but also by ageing and AD, which are correlated with cognitive decline. Indeed, these factors and their receptors are both reduced in the afflicted brain regions of demented subjects and this reduction can be evident prior to symptoms' onset, suggesting that it could cause or accelerate the disease[932]. In addition, lower serum BDNF levels have been detected in elderly women[933] and high concentrations of the same molecule have been found around amyloid plaques, due to the presence of activated microglia and astrocytes[934,935]. It has also been demonstrated that Aβ is able to alter TrkB isoforms, which are also regulated by PS-1 gene[936] and that a reduction in BDNF retrograde axonal transport is present in AD mouse models[937]. Moreover, some polymorphisms in neurotrophins genes have been identified as risk factors[938]. Indeed, carriers of the Met BDNF allele have shown increased brain atrophy and faster cognitive decline[939] whereas carriers of both Met BDNF allele and ApoEε4 are characterized by enhanced neurodegeneration[940].

Not only BDNF but also NGF seems involved in dementia. Indeed, the cognitive decline is due to the deterioration of the basal forebrain cholinergic neurons, which are responsible for the modulation of memory, learning and attention[941]. These cells are highly vulnerable and affected in AD[942,943] and continuously depend on NGF during post-natal stages[944]. They are the major cellular group expressing this neurotrophin together with its TrkA receptors in the CNS and this high expression is also typical of their projection areas[945] and is essential for their survival during development[946]. Given the important role of this molecule in their maintenance, it was hypothesized that their damage in AD could depend on NGF deficits[947]. Thus, the exogenous neurotrophin was employed in some animal experiments and clinical trials but, even though the treatment succeeded in rescuing basal forebrain cholinergic neurons' down-regulation and atrophy[948,949], many side effects and complications arose, making this therapy not suitable[950,951]. In addition, it was demonstrated that mature NGF mRNA levels do not change during AD and pro-NGF quantity is even increased[952,953], together with the activity of NGF-degrading enzyme, whereas TrkA receptors are deeply reduced[954]. On the contrary, p75NTR expression is stimulated by amyloid peptide, which may mimic NGF functions and could decrease its own aggregation due to NGF receptors over-expression[955]. The same alterations were observed in MCI patients and also following Aβ oligomers injection in the brain, suggesting that NGF dysfunction could be a an early sign of the disease[956]. Moreover, lack of mature NGF causes atrophy, loss of cholinergic neurons

and amyloid plaques deposition[957,958]. On the contrary, in aged animals NGF was found to be highly peroxynitrated and therefore biologically inactive, indicating that its deficits might not depend on a failure in its extracellular metabolism. The increased NGF brain levels are mirrored by higher concentrations of this molecule in the CSF of AD patients, although it is not clear if this increase is due to the mature or the precursor form[959]. Furthermore, some NGF polymorphisms have been associated to early-onset or late-onset AD but these results haven't been definitively confirmed yet[960].

Considering neurotrophins involvement in AD, these molecules might have a therapeutic effect, because they are able to restore cognitive functions without reducing neuropathology[961]. In particular, BDNF has been shown to protect neurons from cytotoxic Aβ fragments[962] and also to inhibit β-amyloid production by inducing the expression of SORLA protein, which reduces APP processing and plaques formation[963]. Moreover, it is able to reduce tau hyper-phosphorylation[964] and prevents septal cholinergic neurons from dying[965]. Given its pharmacological properties, different strategies to counteract AD have been based on enhancing BDNF signalling, either by the direct administration of the exogenous compound or by the activation of its receptors[966]. On the other hand, NGF has been shown to activate the APP non-amyloidogenic processing pathway, to reduce Aβ production[967] and its concentration in CSF of AD patients[968]. However, its employment has been correlated to aberrant neurite sprouting[969] and increased tau hyper-phosphorylation[970].

Thus, there are no effective treatments to reverse the pathology and different reasons account for neurotrophins' failures in clinical trials, such as limited access to the CNS and short biological half-life[971]. Recently, some neurotrophins' receptors agonists have been developed to bypass these problems but targeting the appropriate cell type, without affecting healthy tissue, is still challenging[972]. Other strategies aim to enhance trophic factors' expression but they are not used as a routine therapy, because both delivery and induction of these molecules require invasive surgery and causes neuropathic pain.

4.6 Neurotrophins and BPSD

Beyond their role in neuroinflammation and AD, neurotrophins have also been linked to neuropsychiatric disturbances. Indeed, reduced BDNF levels have been found in brains from

depressed and suicidal victims[973], as well as in animal models of depression[974]. In support of these observations, BDNF has been shown to have a role in the treatment of patients suffering from major depression[975]. Since inflammation might contribute to the development of neuropsychiatric diseases[461,976] and given BDNF involvement in these pathologies, their presence have been attributed to the activation of the immune system. In agreement with this hypothesis, LPS brain infusion induces anxious and depressed behaviour, which is associated to decreased BDNF expression[977]. Instead, the levels of this neurotrophin are up-regulated following the administration of anti-depressant drugs[978,979] and this molecule, together with other neurotrophins, can be considered a valid biomarker reflecting emotional state[980]. Similarly, higher concentrations of BDNF have been found in schizophrenia and bipolar disease patients[981,982] and also aggressiveness was positively correlated with plasma BDNF levels[983].

Moreover, since this molecule has neurotrophic functions also on motor-neurons, its down-regulation could account for ALS neurodegeneration[984], which has been linked to immune alterations as well. Indeed, it has been demonstrated that BDNF dysfunction might be responsible for depressed mood and severe functional loss in ALS patients, strengthening its role in influencing both the nervous and the immune system[985].

Thus, neurotrophins have important roles in maintaining homeostasis and ensuring the correct development of the CNS. Since they are similarly affected by neuroinflammation, AD and neuropsychiatric disturbances, it is reasonable to hypothesize that the three processes are deeply connected and might refer to several common pathological pathways.

Chapter 5

Aim

5.1 TSPO/DBI System (part 1)

➢ Scientific Background

Nowadays, AD is recognized as the third leading cause of death and the number of affected subjects is continuously rising, together with population ageing. The most critical aspect of this pathology is represented by BPSD, which often lead to the premature institutionalization of patients, increasing the costs of health care. This early hospitalization is especially due to disturbances such as psychosis, aggression and agitation, which need to be treated by qualified staff. Indeed, dealing with this kind of symptoms is very difficult for the caregivers, who consequently experience high stress burdens if they have to manage the diseased subjects without the help of clinical experts. To date, BPSD biological causes have not been clarified yet. Therefore, pharmacological treatments are only symptomatic and, most of the time, they display severe side effects that make their use more limited.

Even though the mechanisms underlying these disturbances are still unknown, multiple evidences seem to relate behavioural anomalies to immune system dysregulation. In support of this hypothesis, many studies have demonstrated that systemic infections can cause non-cognitive symptoms very similar to the ones observed in AD. Indeed, in a rat model, LPS-induced sepsis gave rise to long-term consequences, which include behavioural deficits, neuronal loss and reduced cholinergic innervation[986] and the same outcome was described before, in a model of neuroinflammation induced by both acute and chronic LPS injections[987,988]. Thus, peripheral infections have been demonstrated to account for behavioural and emotional changes, which are often experienced by not only inflamed subjects but also AD patients.

Another evidence for the involvement of the immune system in the genesis of non-cognitive disturbances derives from a clinical condition known as delirium that is characterized by neuropsychiatric symptoms very similar to BPSD. Its causes have not been established yet but several studies demonstrated that its pathogenesis might be correlated to a higher production of pro-inflammatory mediators, which are no longer balanced by the anti-inflammatory ones[989]. Indeed, systemic LPS injections can trigger exaggerated CNS inflammation and sickness behaviour responses[990,991], which might represent predisposing factors to future dementia.

Thus, systemic inflammatory conditions as well as neuroinflammation seem to be somehow

related to behavioural disturbances, although the underlying mechanisms have not been elucidated yet. In this context, our interest concentrated on the TSPO/DBI system. Indeed, these elements are both highly expressed in brain and CSF of AD patients[992,993] but they are also detected in periphery, where TSPO densities on PBMC plasma membrane are modulated by stress as well as anxiety[994–998]. Also, the severity index of this symptom has been correlated to the concentration of this receptor on platelets membrane[999] and, additionally, it accounts for the impaired ability of this transporter to regulate monocytes chemotaxis[1000,1001]. Moreover, a decreased TSPO expression has been found in both PBMC and platelets of patients suffering from schizophrenia and post-traumatic stress disorder[1002,1003], whereas its increased levels have been associated to the occurrence of depression[1004]. Finally, some polymorphisms in the TSPO gene have been linked to panic disorder[1005], strengthening the possible link between this receptor and behavioural anomalies.

This relationship is further supported by the role of TSPO in neurosteroids production. Indeed, it represents a cholesterol transporter to move this precursor from the cytoplasm to the mitochondria[1006–1008] and start neurosteroidogenesis. Interestingly, neurosteroids are either agonists or antagonists of the $GABA_A$ receptors in the CNS but they also exert their functions in the peripheral nervous system[1009–1021]. Here, altered levels of these molecules have been measured during ageing or inflammation[1022–1025] and their dysregulation has been linked to stress and behavioural complications, such as depression, anxiety, post-traumatic stress disorders and schizophrenia[1026–1035]. Nevertheless, these anomalies have been attributed either to increased or decreased concentrations of neurosteroids, implicating that they might have different effects according to the environment and the pathway they activate. On the other hand, some of these molecules have been shown to be altered in AD patients[295,1036,1037], in which an up-regulation of neurosteroidogenic enzymes has been also detected[1038], together with increased DBI CSF levels[1039]. These evidences underline neurosteroids influence on the genesis of behavioural disturbances and reinforce the hypothesis of a possible role for the TSPO/DBI system in mediating these effects.

➢ Aim of the study

In light of the complex scenario pictured above, the main aim of this study is to characterize the TSPO/DBI system, as regards its neurosteroidogenic role, in AD patients as well as in healthy

controls, in order to unravel potential alterations that might account for behavioural manifestations in the diseased cohort.

For this purpose, we took advantage of the peripheral localization of this system, given that reaching it into the CNS is complicated due to the presence of the BBB. However, the bi-directional communication existing between brain and periphery provides the possibility to study central alterations at a systemic level. Thus, we collected whole blood samples from our subjects and we processed them to isolate both PBMC and serum to perform our analyses. In order to examine the TSPO/DBI system at different levels, we decided to evaluate several parameters, such as TSPO gene expression, DBI and BDNF serum levels and CDR. The reason why we chose BDNF lies into the neurosteroidogenic pathway, indeed this neurotrophin is a down-stream product of TSPO activation and, similarly, CDR is a ratio deeply influenced by DHEA-S, which is a by-product of the same process.

To investigate more in depth the putative role of the TSPO/DBI system in the genesis of behavioural anomalies, we screened our AD patients for the presence of the agitation/aggression symptom, which is the most difficult to manage for caregivers and also the most frequent cause of institutionalization. In this cohort, we performed the same analyses listed in the paragraph above, hoping to unravel a potential marker that could identify this disturbance. Moreover, given that the subjects displaying this anomaly are also characterized by motor hyperactivity, we also examined their irisin serum levels, since this molecule has been related to physical activity as well as BDNF production, providing a link to the TSPO/DBI system.

On the other hand, we concentrated on two single-nucleotide polymorphisms, rs6971 in the TSPO gene and rs6265 in the BDNF one, that have been correlated to mood disturbances and BDNF production, respectively. We were interested in analysing the putative influence of these SNPs on the TSPO/DBI system and, for this aim, we examined the previous data collected in our AD subjects, dividing them according to their genotypes.

All the parameters we investigated were correlated to a panel of clinical, demographic and social data regarding the patients, in order to highlight any possible influence of these factors on pathological manifestations.

Furthermore, we explored if the potential modifications in the TSPO/DBI system are specific for amyloidopathy (AD) or if they are present in other pathogenic contexts characterized by cognitive

impairment. To do so, we enrolled subjects affected by an amnesic Mild Cognitive Impairment (aMCI) as well as other forms of cognitive deterioration (NDCI) and we screened both these populations for DBI serum levels. In the second cohort, we also measured the concentration of this molecule in the CSF, to verify the possible existence of a correlation in DBI levels between this biological fluid and serum. On the other hand, we also examined DBI serum levels in a few subjects affected by delirium, which is known to lead to behavioural disturbances similar to BPSD. Moreover, in these individuals we assessed the serum concentration of two pro-inflammatory cytokines (TNF-α and IL-6), which are supposed to be increased, given the strong systemic inflammatory response typical of delirium clinical condition.

In case of neuroinflammation, an increased motility of peripheral cells towards the CNS has been reported by several studies and seems to boost the central inflammatory process. Since TSPO has been shown to regulate monocytes migration, we also examined the potential impact of TSPO/DBI system alterations on this specific cellular function. For this purpose, we performed two types of chemotaxis assays using a THP-1 monocyte-like cell line[1040] as well as peripheral monocytes from AD patients and healthy controls and we stimulated them with different substances, such as Aβ. Indeed, this peptide has a central role in AD pathology and we were interested in verifying if it could represent a disease-specific trigger for peripheral monocytes to migrate towards amyloid deposition sites. Moreover, we exposed our cells to the compound Ro5-4864, which is known to inhibit the TSPO receptor, in order to see if this inhibition could influence monocytes motility.

Thus, in the first part of this study we tried to identify possible alterations of the TSPO/DBI peripheral system which could account for either variations in neurosteroids levels or in monocytes chemotaxis that, in turn, might be responsible for the behavioural anomalies observed in AD patients. Moreover, we tried to detect a biological marker among the parameters we analysed, so that it could be used to characterize behaviourally-disturbed subjects in clinical trials. Indeed, the actual available instruments are not totally reliable because they depend on the opinion of the caregivers and not directly on the patients themselves.

5.2 Neutrophils (part 2)

➢ Scientific Background

The role of neuroinflammation in AD pathogenesis has already been recognized but only recently neutrophils have arisen as important elements of this complex scenario. Indeed, these cells have been found in brain vessels and parenchyma of both 3xTg-AD and 5xFAD mouse models, where they seem to release intravascular as well as intraparenchymal NETs[1041]. These structures are endowed with the ability to eliminate pathogens but they might also induce cytokines over-production and NLRP3 inflammasome activation, leading to neuronal damage. Moreover, neutrophils have been shown to extravasate from systemic circulation via an LFA-1/ICAM-dependent mechanism and, in the CNS, they have been detected in close proximity to amyloid plaques[1041]. Indeed, MPO+ cells have been identified in these areas and this labelling has been confirmed to co-localize with NE and citrullinated histone H3, which are both markers of NETs.

Similar results have been observed in AD patients, which display activated neutrophils not only in brain blood vessels but also near Aβ deposits, reinforcing the important role of these cells and their transmigration in disease pathogenesis[1041–1044].

➢ Aim of the study

Considering the findings described in the paragraph above, the aim of the second part of this study is to search for the putative presence of infiltrating neutrophils in a context of NLRP3 inflammasome deficiency superimposed on a genetic AD condition as well as in the genetic AD pathology per se.

For this purpose, we analysed the brains of 5-month-old (young) and 15-month-old (old) mice, belonging to four different genetic backgrounds, which include wild type (WT), APP/PS1 (AD mouse model), *Nlrp3*$^{-/-}$ (wild-type mouse deficient for the inflammasome) and APP/PS1/*Nlrp3*$^{-/-}$ (AD mouse model deficient for the inflammasome). Moreover, every age and genotype were considered in an untreated control condition (CTRL), 2 days (2d) and 10 days (10d) after one single intraperitoneal LPS injection that was aimed to activate a strong inflammatory response. Nevertheless, given that this bacterial PAMP represents a trigger for NLRP3 inflammasome, animals deficient for this complex were expected to display a very mild inflammation. After

animals were sacrificed, their brains were cut and the slices were preserved in sodium azide before performing immunohistochemistry.

All the samples were stained for microglia and amyloid deposits as well as for activated neutrophils and we analysed both plaque-associated and plaque-free areas, in order to detect any possible variation depending on Aβ presence. Also, we manually counted the positive cells in our brain slices to highlight any potential difference deriving from the genetic background or the age.

Thus, the overall goal is to shed light on neutrophils role not only in a mouse model of Alzheimer's Disease but also in a particular background of inflammasome deficiency, which has previously showed beneficial effects in ameliorating cognitive deficits typical of this neurodegenerative pathology[1045].

Chapter 6

Materials and Methods

6.1 Main Study

➢ Recruited subjects

Following ethical approval and informed consent, AD patients were recruited at the Alzheimer Evaluative Unit *(UVA, Unità Valutativa Alzheimer)* of the Neurological Clinic at *San Gerardo* Hospital (Monza, Italy), with the collaboration of the Neurological Clinic at *A. Manzoni* Hospital (Lecco, Italy). The subjects were diagnosed *probable* dementia and Alzheimer's disease according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) as well as of the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA), respectively.

Following ethical approval and informed consent, the aMCI subjects were initially characterized by a clinical and psychometric evaluation. The diagnosis was confirmed by means of MRI and FDG-PET. The subjects underwent blood withdrawal and Flutemetamol-PET during pharmacological trials in the *San Gerardo* Hospital, before the start of experimental drugs therapy.

The NCDI cohort was selected among the patients of the Alzheimer Evaluative Unit *(UVA, Unità Valutativa Alzheimer)* of the Neurological Clinic at *San Gerardo* Hospital (Monza, Italy) as well as from the patients institutionalized in the same hospital. These subjects were evaluated by means of clinical, psychometric and imaging (MRI and PET) assessments but their population was heterogeneous, including individuals with deficits ascribable to different types of dementia involving frontal functions (executive or linguistic). The differential diagnosis was set between frontal-expressed AD vs. FTD, CBS or PSP. For this reason, these patients underwent blood withdrawal and lumbar puncture to define their protein accumulation profile (Aβ vs. Tau).

Healthy controls were age- and sex-matched with AD subjects and they were recruited among patients' relatives at the Neurological Clinic of the *San Gerardo* Hospital (Monza, Italy), following informed consent. The exclusion criteria to select this cohort were as the same as the ones employed to select the AD population. All the recruited subjects had a sedentary life-style and they did not practice sport or regular physical activity.

Inclusion criteria

The patients included in the study had a *probable* AD diagnosis according to the DSM-V and NINCDS-ADRDA criteria as well as an MMSE ≤ 26. The healthy controls had an MMSE > 26 and were available to undergo blood withdrawal.

Exclusion criteria

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

- positive anamnesis for major cranial trauma
- chronic intestinal inflammatory diseases
- hypo- and hyperthyroidism
- kidney or liver insufficiency
- abuse of alcohol, drugs or other substances
- major depressive disorder
- malignant neoplasms, especially the ones involving lungs, ovary, pancreas and blood

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 listed:

- corticosteroids
- immune-suppressors
- anti-neoplastic drugs

Cognitive deterioration and previous or on-going neurologic/psychiatric pathologies were also considered exclusion criteria to recruit healthy controls.

Patients' examination

Enrolled AD subjects were interviewed as regards clinical and pharmacological anamnesis and they were administered the following clinical tests:

- Mini-Mental State Examination (MMSE) to evaluate their global cognitive performances[1046]
- Neuro-Psychiatric Inventory (NPI) to evaluate their neuropsychiatric symptoms and the distress of their caregivers[284]
- Caregiver Burden Inventory (CBI) to evaluate caregivers' stress burden[1047]

➢ PBMC and serum samples preparation

Every subject in this study underwent blood withdrawal (15 ml) to collect 3 different aliquots:

- whole blood in K_3EDTA to obtain PBMC pellet (5 ml)
- whole blood in K_3EDTA to perform genetic analyses (5 ml)
- whole blood without anti-coagulant to obtain serum (5 ml)

PBMC separation

Whole blood was diluted 1:1 with physiologic solution (NaCl), layered on an equal volume of density gradient medium (Lympholyte) and centrifuged (490 ×*g*, 30 min, 18 °C). The PBMC were collected at the plasma-Lympholyte interface, washed in physiologic solution and stored at -80 °C.

Serum separation

After 1 h incubation at 37 °C, whole blood was centrifuged (2800 ×*g*, 15 min, RT). Serum was collected and stored at -80 °C.

➢ CSF samples preparation

The NDCI subjects underwent lumbar puncture with a 21-gauge needle to extract CSF (5 ml), which was collected in a polypropylene tube. Part of the CSF sample was used to perform routine analyses (leucocytes and erythrocytes count, glucose and total proteins concentration), whereas the remaining was centrifuged (2000 ×*g*, 10 min, RT) and stored at -80 °C.

➢ DBI concentration measurement

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

➢ CSF measurement of $A\beta_{1-42}$, t-tau and p-tau

The CSF $A\beta_{1-42}$, t-tau and p-tau concentrations were measured using commercially available kits for diagnostics (Innotest β-amyloid 1-42; Innotest hTAU-Ag; Innotest Phosphotau (181P) – Fujirebio, Europe Ghent, Belgium), following the manufacturer instructions.

➢ RNA extraction and cDNA synthesis

Total RNA was extracted from PBMC using the RNeasy Mini kit (Qiagen), according to the manufacturer instructions. RNA concentration was determined spectrophotometrically at 260 nm. cDNA was synthesized from RNA using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen®) at the following conditions: 10 min at 25 °C and 60 min at 42 °C. The reaction was terminated at 85 °C for 5 min and cDNAs were stored at -20 °C.

➢ Real-time PCR (qPCR)

For the analysis of TSPO mRNA levels, the cDNAs obtained from 1500 ng RNA were amplified in duplicate in the ABI Prism 7500 HTSequence Detection System (Applied Biosystems™) using TaqMan® Gene Expression Assay (Applied Biosystems™, TSPO assay ID: Hs00559362_m1; β-actin assay ID: Hs99999903_m1) at the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of: 95 °C for 15 s and 60 °C for 60 s. For relative quantification of the target vs. β-actin mRNA, the comparative C_T method was used as previously described[1048].

➢ DNA extraction and genotyping

Total DNA was extracted from whole blood using the QIAamp DNA Blood Mini kit (Qiagen), according to the manufacturer instructions and DNA concentration was determined spectrophotometrically at 260 nm. Then, 200 ng DNA were amplified in a thermo-cycler at the following conditions: 94 °C for 5 min, 35 cycles of: 94 °C for 30 s and 67 °C (TSPO) or 72 °C (BDNF) for 30 s, 72 °C for 7 min.

The following primer pairs (Sigma-Aldrich) were used: TSPO-F (CCTTGGTGGATCTCCTGCTGGT) and TSPO-R (ACATCACAAGCGTGATGGCACC); BDNF-F (AAAGAAGCAAACATCCGAGGACAAG) and BDNF-R (ATTCCTCCAGCAGAAAGAGAAGAGG). Each DNA sample was loaded on 2% agarose gel to evaluate DNA quality and genotyped using Sanger sequencing method in the laboratory directed by Prof.

Romina Combi (University of Milan-Bicocca, Monza, Italy). The data were analysed using ChromasPro v.1.34 software (Technelysium Ltd, South Brisbane, Australia).

➢ ApoE genotyping

The ApoE genotyping was performed digesting DNA with restriction enzymes (HhaI) and loading the amplification product of this digestion on 4% agarose gel, according to[1049].

➢ CDR quantification

Serum DHEA-S and cortisol levels were determined by means of electrochemiluminescence (Cobas C6000 E601, Roche Diagnostics, Mannheim, Germany) in the analyses laboratory of the *San Gerardo* Hospital (Monza, Italy). CDR was calculated as cortisol-to-DHEA-S molar ratio.

➢ BDNF concentration measurement

The serum BDNF concentration was measured using a commercially available kit (Promega BDNF Emax® ImmunoAssay System). The samples were previously diluted 1:128 in distilled water and the assay was performed according to the manufacturer instructions. Briefly, samples were loaded into the plate wells, incubated (2 h, RT) and washed 5 times before adding the primary antibody (2 h, RT). After 5x washes, they were incubated with the secondary antibody (1 h, RT), washed 5 times and incubated with TMB to start the colorimetric reaction, which was stopped after 10 min using HCl 1N. BDNF concentration was determined spectrophotometrically at 450 nm and it was quantified according to the standard curve.

➢ Irisin concentration measurement

The serum irisin concentration was measured using a commercially available kit (Phoenix Pharmaceuticals, Burlingame, CA). The samples were previously diluted 1:5 in distilled water and the assay was performed according to the manufacturer instructions. Briefly, samples were loaded into the plate wells together with the primary antibody, incubated (2 h, RT) and washed 3 times before adding the secondary antibody (1 h, RT). Then, TMB was added to the wells (1h, RT) and the reaction was stopped using HCl 1N. Irisin concentration was determined spectrophotometrically at 450 nm and it was quantified according to the standard curve.

➢ Chemotaxis

The chemotaxis experiments were performed using two different assays.

µ-slide chambers (Ibidi®)

The THP-1 cells were suspended in gel matrix to reach the final concentration of 3×10^6 cells/ml, then 6 µl of this suspension were loaded in the central channel of the µ-slide chamber and set aside until the gel was solid. Later, one lateral chamber was filled with cellular medium alone and the other one with cellular medium and 15 µl of chemoattractant (MCP-1 10 ng/ml or Aβ 125 nM). Cellular migration was recorded by means of time-lapse microscopy and the migratory trajectories of 20 cells/chamber were designed using the Chemotaxis and Migration Tool (Ibidi®). The Forward Migration Index (FMI, parallel component of the chemotactic gradient) was used to measure cellular motility: higher migration rates correspond to higher FMI values.

Boyden chambers (Corning™ Transwell™)

The PBMC pellets obtained from whole blood were resuspended in RPMI cellular medium and incubated at 37 °C for 24 h to separate lymphocytes from monocytes. Adherent monocytes were collected and suspended in cellular medium to reach the final concentration of 5×10^5 cells/ml. 600 µl of each chemoattractant solution (MCP-1 10 ng/ml or Aβ 125 nM, 1.25 nM, 125 pM) or inhibitor solution (Ro5-4864 10 µM, 25 µM, 50 µM) were loaded in the lower chamber of each well and an insert was added to separate this part from the upper one by means of a filter (pores Ø: 5 µm). 100 µl of the cellular suspension were loaded in the upper chamber and the plate was incubated at 37 °C for 90 min. After the incubation, the filters were fixed, coloured with a modified-Giemsa staining, removed from the inserts, mounted on glass slides and observed at the optical microscope (magnification 10x). Nucleated cells in 10 optical fields/filter were counted and their number was used as a chemotactic index.

➢ Animals and ages

All the animals were provided by Michael T. Heneka research group (University of Bonn Medical Centre, Bonn, Germany). APP/PS1 transgenic animals were obtained from The Jackson Laboratory (number 005864) on the C57BL/6 background. NLRP3-deficient animals (Millennium Pharmaceuticals) were backbred onto C57BL/6 mice genotype to more than 99% C57BL/6, which

was confirmed by microsatellite analysis. All mice were housed under standard conditions at 22 °C and a 12 h light:dark cycle with free access to food and water. Animal care and handling was performed according to the Declaration of Helsinki and approved by the local ethical committees. The following animal groups were analysed: WT, *Nlrp3*$^{-/-}$, APP/PS1, APP/PS1/*Nlrp3*$^{-/-}$.

➢ Tissue preparation

Mice were deeply anaesthetized and transcardially perfused with 15 ml phosphate-buffered saline (PBS). The brains were removed from the skull. One hemisphere was frozen immediately for biochemical analysis and the other was either fixed in 4% paraformaldehyde or frozen over a mixture of dry ice and isopentane.

➢ Immunohistochemistry

Free-floating 40 µm serial sections were cut on a vibratome (Leica). Sections were stored in 0.1% NaN_3, PBS. For immunohistochemistry, antigen-retrieval was performed before starting the staining. The slices were immersed in sodium citrate buffer and heated at 90 °C for 30 min. Then, sections were washed three times for 7 min in PBS-T (PBS, 0.1% Triton X-100), blocked in 1% BSA in PBS-T (blocking buffer) for 1 h at RT and incubated O. N. at 4 °C with the primary antibody in blocking buffer. Specificity controls were performed by staining with the secondary reagent and omission of the primary antibodies. Sections were washed three times in PBS-T, incubated with Alexa-488- or Alexa-594-conjugated secondary antibodies (1:500, Invitrogen®), together with methoxy-X04 (1:1000, Abcam) and DAPI (1:1000) for 1 h at 37 °C, incubated with 1% Sudan Black for 20 min at RT and washed three times with PBS for 10 min. Sections were mounted using Immomount (Thermo). The following primary antibodies were used with respective concentrations: goat polyclonal anti-MPO (1:20, R&D Systems), rabbit polyclonal anti-NE (1:100, Abcam), rat monoclonal anti-GFAP (1:1000, Invitrogen®), rabbit polyclonal anti-Iba1 (1:400, Wako), goat polyclonal anti-Iba1 (1:400, Novus), rabbit polyclonal anti-Ki67 (1:1000, Abcam). Fluorescence microscopy was performed on an Olympus BX61 equipped with a spinning disk unit, using Cell P 3.5 software (Olympus). The final processing was done using Fiji (ImageJ software).

➢ Statistical analyses

Statistical analyses were performed using Prism 7 (GraphPad Software). Data are expressed throughout the study as mean ± standard deviation (SD). 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. Correlation was computed with the two tailed Pearson's r-test. The significance of differences in frequency between two groups was evaluated by means of the $\chi 2$ test. Values that differ by two standard deviations from the mean value of each group were excluded from the analysis.

6.2 Appendix

➢ Recruited subjects

Following ethical approval and informed consent, ALS patients were recruited at NEMO Clinical Centre of the *Niguarda* Hospital (Milan, Italy), with the collaboration of the Neurological Clinic at the *San Gerardo* Hospital (Monza, Italy). The subjects were diagnosed sporadic ALS (probable or defined) according to El Escorial Criteria[1050] and they were all home-nursed by their main caregivers, which were interviewed to collect information about patients' behavioural disturbances. Beyond being administered ALSFRS-r, DPI, FAB[1051] and MoCA[153], the patients were screened for demographic, social and clinical data listed in **Table 7**. Liver and kidney functionality was standard for every subject, including the ones treated with Riluzole.

Healthy controls were matched for age and sex with ALS patients and they were recruited among patients' relatives at the Neurological Clinic of the *San Gerardo* Hospital (Monza, Italy), following informed consent.

Cancer as well as autoimmune or inflammatory diseases were considered exclusion criteria. Healthy controls were not affected by any other neurological or psychiatric pathology and they were not in treatment with psychoactive drugs. Their MMSE was always >26.

Chapter 7

Results (part 1)

7.1 Alzheimer's Disease

➢ Recruited population

For the first part of this work, we recruited n=60 Alzheimer's Disease patients (AD), which were diagnosed the pathology according to the NINCDS/ADRDA as well as DSM-V criteria and that were characterized for many different parameters listed in **Table 1**. As underlined by the aim of this project, we are particularly interested in behavioural manifestations, which are present in 93% of our diseased individuals. To strengthen our analyses, we considered agitation/aggression (AA) not only as a domain of the NPI-12 test but also as an independent index, in order to evaluate the percentage of patients displaying this symptom (40% in our population). Moreover, we extrapolated its specific score (AA score = frequency × severity) and we calculated the time lag between the onset of the pathology and the earliest appearance of these disturbances (O/AA-lag).

In parallel, we recruited n=30 non-demented healthy controls (CTRL), matched with the diseased subjects for age and sex.

Both populations were screened for markers belonging to the TSPO/DBI peripheral system, in order to highlight potential differences between the two cohorts.

➢ BPSD distribution, clustering and severity

Analysing the AD population for BPSD distribution, we observed that the 93% of our patients displayed a positive NPI-12 score. The most frequent registered symptom in these subjects was apathy (73%), followed by depression (48.6%) and agitation/aggression (47.5%) (**Fig. 1**).

Moreover, grouping the behavioural manifestations in clusters, we established that 19% of AD patients were ascribable to one cluster only, whereas 27% to two, 30% to three and 24% to four of them (**Fig. 2**). Also, considering the whole population, 81% of diseased subjects displayed symptoms belonging to more than one cluster, even though it was always possible to determine the predominant one.

	AD (n=60)					CTRL (n=30)	
Sex (% M)	25 (42%)					14 (47%)	n. s.
Age (ys)	78 ± 6 (69-88)					79 ± 6 (66-86)	n. s.
Level of education (ys)	6 ± 2.7 (3-13)					N/D	N/A
Disease duration (mo)	39.8 ± 24.3 (4-96)					N/A	
MMSE (score)	17.79 ± 5.7 (4-26)					>26	p<0.001
CDR 1 / 2 / 3 (%)	57% / 36% / 7 %					N/A	
BPSD+ (n, %)	55 (92%)					N/A	
NPI-12 (score)	18.49 ± 2.31 (0-86)					N/A	
AA+ (n, %)	24 (40%)					N/A	
AA (score)	5.8 ± 3.4 (0-12)					N/A	
O/AA-lag (mo)	27.1 ± 26.3* (0-79)					N/A	*median, interquartile range: 19, 0-56
CBI (score)	24 ± 19 (0-61)					N/A	
Social support (%)	Familiar assistant: 17% NHC facility: 4%					N/A	
Antipsychotic therapy (%)	25%					N/A	
Haloperidol equivalents	0.69 ± 0.41 (0.16-1.48)					N/A	n=12 (20%)
AChEI (n, %)	44 (74%)					N/A	
Memantine (n, %)	21 (35%)					N/A	
ApoE genotype (%)	**2/2**	**2/3**	**3/3**	**3/4**	**4/4**	N/P	
	2%	5%	39%	44%	10%		

Table 1: Complete list of the clinical, demographic and social information about AD and CTRL recruited populations. The social support refers to non-domestic help, such as a family assistant or a nursing home care (NHC) facility. The antipsychotics dosage was expressed in terms of haloperidol equivalents[1052] (two-tailed Student's t-test, mean ± SD) (N/A: not applicable; N/D: non determined; range intervals in brackets).

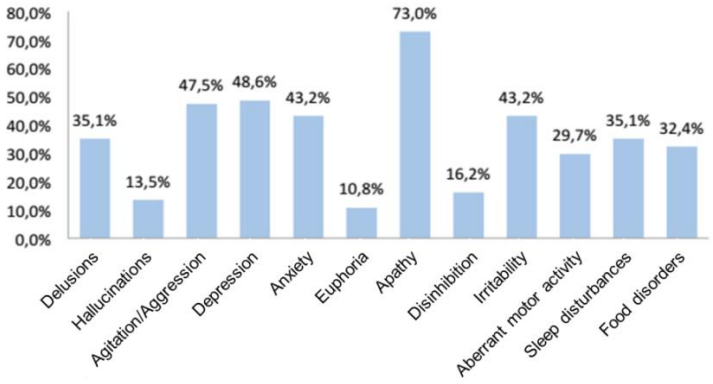

Figure 1: BPSD distribution in the recruited AD population. The NPI-12 score is positive in 93% of the patients and the main symptoms they display are apathy, depression and agitation/aggression.

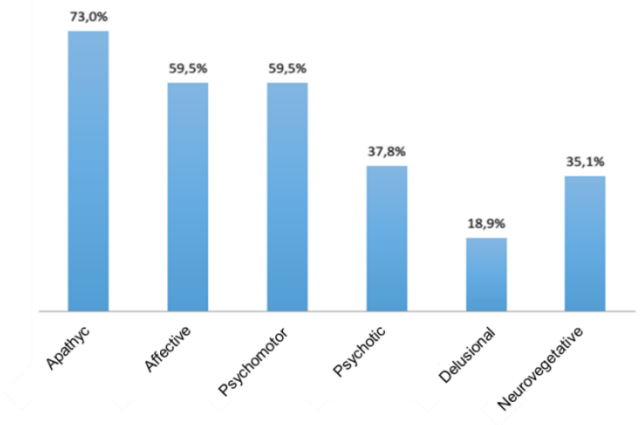

Figure 2: Clusters of BPSD in our AD patients population. The frequency of the psychomotor cluster mainly derives from the agitation/aggression domain (63%) and it is only partially influenced by the aberrant motor behaviour one (37%).

As regards clinical data concerning BPSD severity, we detected an inverse correlation between NPI-12 and MMSE scores (r=-0.50, p<0.001) (**Fig. 3A**). On the contrary, NPI-12 score was directly correlated to the CBI one (r=0.60, p<0.001) (**Fig. 3B**) as well as to disease duration (r=0.46, p<0.001) (**Fig. 3C**).

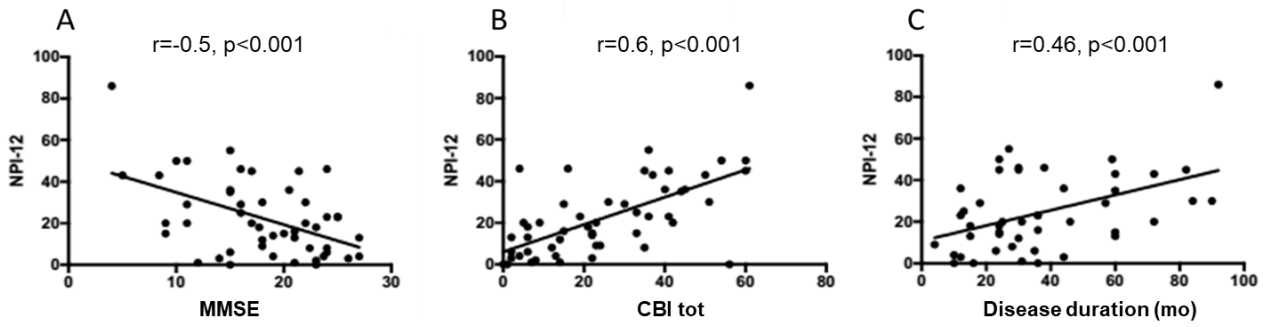

Figure 3: Correlations between clinical data. (A) Inverse correlation between NPI-12 and MMSE scores. Worse BPSD manifestations correspond to a more severe cognitive impairment (**p<0.001). **(B) Direct correlation between NPI-12 and CBI scores.** More severe BPSD disturbances are accompanied by higher stress burdens for the caregivers (**p<0.001). **(C) Direct correlation between NPI-12 score and disease duration.** Behavioural symptoms increase together with disease duration and patients affected for longer times display worse BPSD (**p<0.001).

➢ DBI serum levels

Measuring the serum DBI concentration in both AD and CTRL populations, we observed a significant 118% increase of this value in the diseased cohort compared to the healthy one (39.9 ±

17.0 vs. 18.3 ± 8.3 ng/ml, p<0.0001) (**Fig. 4**). As for TSPO gene expression, no differences emerged after stratifying the patients according to dementia severity, pharmacological therapy or ApoE genotype.

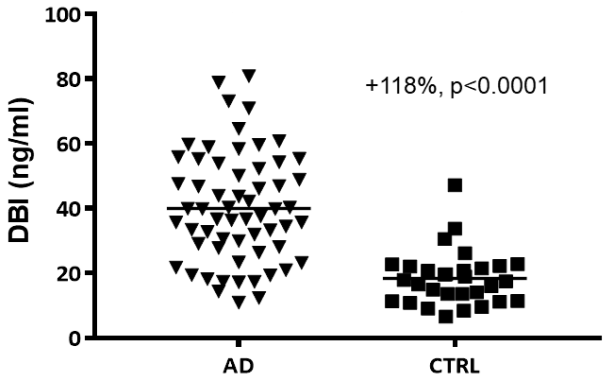

Figure 4: DBI serum levels in AD patients and healthy controls. DBI concentration is increased by 118% in AD patients compared to CTRL (***p<0.0001, two-tailed Student's t-test, mean ± SD).

➢ TSPO expression in PBMC

We evaluated the expression of the TSPO gene in PBMC isolated from the whole blood of both AD and CTRL subjects and we did not find significant changes in its mRNA levels between the two groups (1.30 ± 1.0 vs. 1.70 ± 2.70 RQ, n.s.) (**Fig. 5**). Similarly, no differences emerged after stratifying the patients according to dementia severity, pharmacological therapy or ApoE genotype.

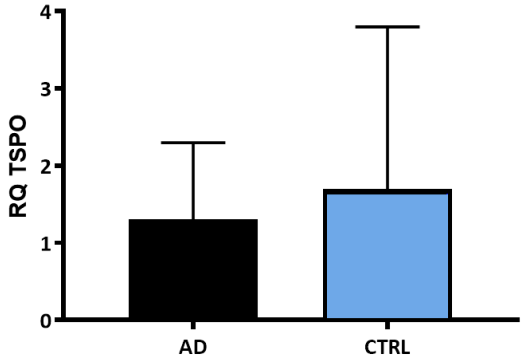

Figure 5: TSPO gene expression in PBMC of AD patients and healthy controls. TSPO gene expression is unchanged between the two groups. Relative Quantification (RQ) of TSPO mRNA is calculated as ratio to β-actin (two-tailed Student's t-test, mean ± SD).

➢ Markers of the TSPO/DBI peripheral system

We analysed the amount of serum BDNF as well as Cortisol-to-DHEA-S Ratio (CDR), which are linked to TSPO activation. Indeed, the first one represents an end product of this process, whereas the second one is a ratio strictly dependent on the concentration of DHEA-S, which is considered a by-product of TSPO stimulation. However, comparing AD to CTRL group, there were significant differences neither in BDNF serum levels (**Fig. 6**) nor in CDR (**Fig. 7**). Moreover, no changes in these markers arose after stratifying the patients according to dementia severity, pharmacological therapy or ApoE genotype.

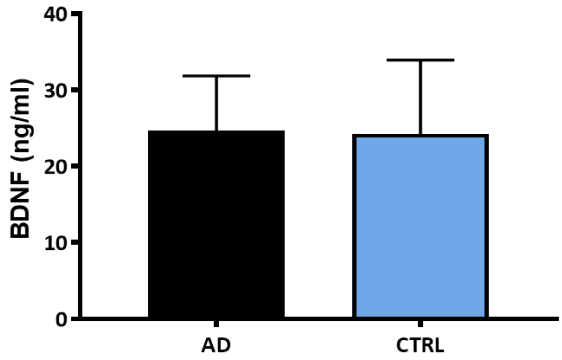

Figure 6: BDNF serum levels in AD patients and healthy controls. BDNF is unchanged between the two groups (two-tailed Student's t-test, mean ± SD).

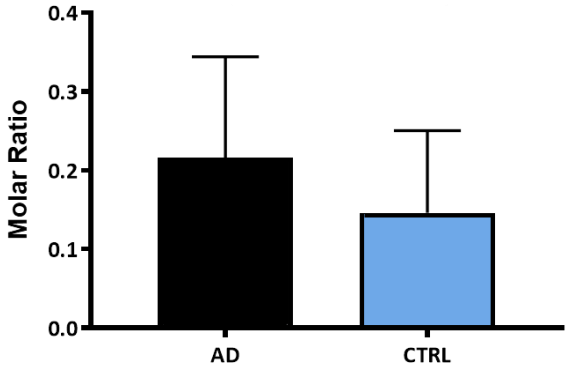

Figure 7: CDR in AD patients and healthy controls. CDR is unchanged between the two groups. The molar ratio is calculated to cortisol (two-tailed Student's t-test, mean ± SD).

➢ Dichotomization of AD patients according to AA

We used the presence of the agitation/aggression (AA) symptom to identify in our AD population a subgroup of subjects called AA+ (AA score > 0), which we contrasted with other patients not displaying this disturbance and, thus, called AA- (AA score = 0). Their full characterization is shown in **Table 2**.

	AD AA+ (n=24)	AD AA- (n=36)	
Sex (% M)	13 (53%)	20 (55%)	n. s.
Age (ys)	78.7 ± 6 (69-85)	77.9 ± 4 (67-70)	n. s.
Level of education (ys)	5.5 ± 2.0 (3-13)	6.3 ± 2.4 (3-13)	n. s.
Disease duration (mo)	46.5 ± 27.0 (12-92)	34.5 ± 21.0 (10-96)	n. s.
MMSE (score)	16.0 ± 6.4 (4-24)	18.6 ± 4.8 (9-26)	p<0.03
NPI-12 (score)	37.17 ± 16.67 (16-86)	13.48 ± 12.42 (0-46)	p<0.0001
AA (score)	6.1 ± 3.2 (1-12)	N/A	N/A
O/AA-lag (mo)	27.8 ± 26.2 (0-79)	N/A	N/A
CBI (score)	37.25 ± 15.72 (9-61)	17.2 ± 15.3 (0-56)	p<0.0001
Social support (%)	Familiar assistant: 33% NHC facility: 3%	Familiar assistant: 8.3% NHC facility: 5%	°χ^2 5.7, p<0.05
Antipsychotic therapy (%)	54%	8.3%	°χ^2 9.1, p<0.003
Haloperidol equivalents	0.75 ± 0.42 (0.33-1.48)	0.16; 0.82	
AChEI (n, %)	75%	72%	n. s.
Memantine (n, %)	32%	38%	n. s.

Table 2: Complete list of the clinical, demographic and social information about AA+ and AA- patients in the recruited AD population. The social support refers to non-domestic help, such as a family assistant or a nursing home care facility. The antipsychotics dosage was expressed in terms of haloperidol equivalents[1052] (two-tailed Student's t-test except when specified χ2 test, mean ± SD) (*median, interquartile range: 19, 0-56; N/A: not applicable; N/P: not performed; range intervals in brackets).

Comparing the clinical data from both groups, we identified some differences mostly related to cognitive decline and BPSD manifestations. Indeed, the AA+ cohort displays lower MMSE scores (-2.6 point on average, p<0.05) (**Fig. 8**) as well as higher NPI-12 (+37.17%, p<0.001) (**Fig. 9**) and CBI (+216%, p<0.001) (**Fig. 10**) scores than the AA- one. Moreover, AA+ patients more frequently need a family assistant in comparison to the AA- subjects (χ^2 5.7, p<0.05).

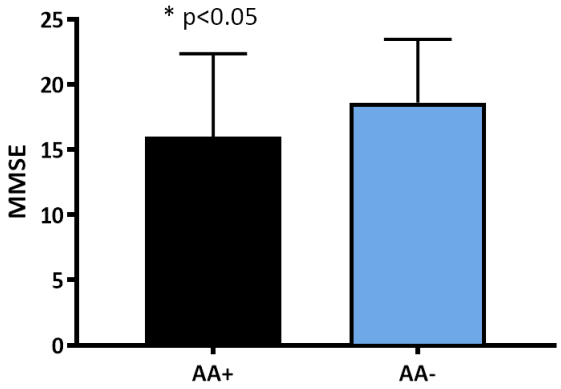

Figure 8: MMSE score in AD patients with (AA+) and without (AA-) agitation/aggression symptom. MMSE score is lower in AA+ patients (*p<0.05, two-tailed Student's t-test, mean ± SD).

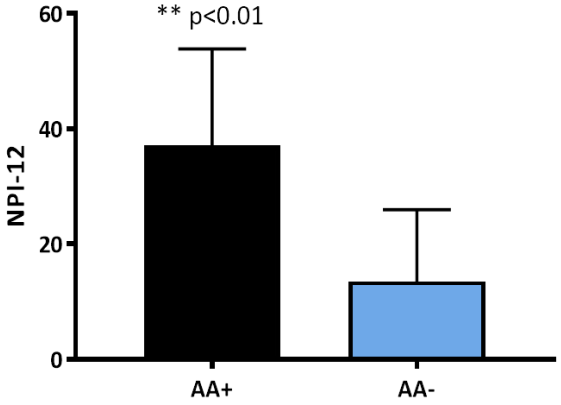

Figure 9: NPI-12 score in AD patients with (AA+) and without (AA-) agitation/aggression symptom. NPI-12 score is higher in AA+ patients (**p<0.01, two-tailed Student's t-test, mean ± SD).

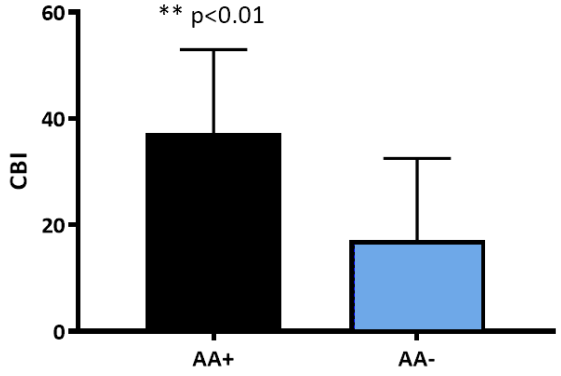

Figure 10: CBI score in AD patients with (AA+) and without (AA-) agitation/aggression symptom. CBI score is higher in AA+ patients (**p<0.01, two-tailed Student's t-test, mean ± SD).

Even though we did not observe any significant difference in the level of education comparing AA+ and AA- subjects, we found an inverse correlation between this parameter and the agitation/aggression NPI score (NPI-AA) in AA+ patients only (r=−0.65, p<0.05) (**Fig. 11**).

In a similar way to what we did in the whole AD population, we screened the AA+ and the AA- subgroups for biological markers of the TSPO/DBI peripheral system.

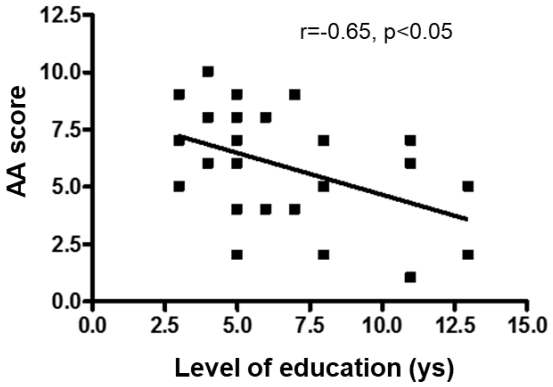

Figure 11: Inverse correlation between the level of education and the NPI-AA score in AA+ patients. Higher NPI-AA scores correspond to lower levels of education (*p<0.05).

➢ TSPO expression in PBMC after AA dichotomization

Considering the expression of the TSPO gene, we detected a modest decrease in AA+ patients compared to AA- ones (1.36 ± 0.70 vs. 1.67 ± 0.80 RQ, p<0.05) (**Fig. 12**). However, other parameters such as serum DBI, serum BDNF and CDR were unchanged between the two groups (data not shown).

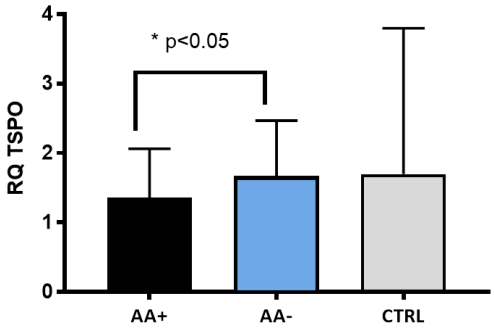

Figure 12: TSPO gene expression in PBMC of AA+ and AA- patients as well as healthy controls. TSPO gene expression is slightly lower in AA+ subjects. Relative Quantification (RQ) of TSPO mRNA is calculated as ratio to β-actin (*p<0.05, two-tailed Student's t-test, mean ± SD).

➢ Irisin serum levels

The AA domain is typically characterized by an active motor behaviour and irisin production has been shown to be influenced by physical activity, thus we measured the serum concentration of this peptide in our AD subpopulations to check if it could be influenced by AA disturbances. We found a slight increase in irisin serum levels in the AA+ subgroup compared to the AA- one (20.5 ± 3.40 vs. 18.6 ± 2.10 ng/ml, $p<0.05$) (**Fig. 13**) but, on the contrary, there were no differences between AD and CTRL cohorts (19.3 ± 2.70 vs. 19.8 ± 3.30 ng/ml, n.s.).

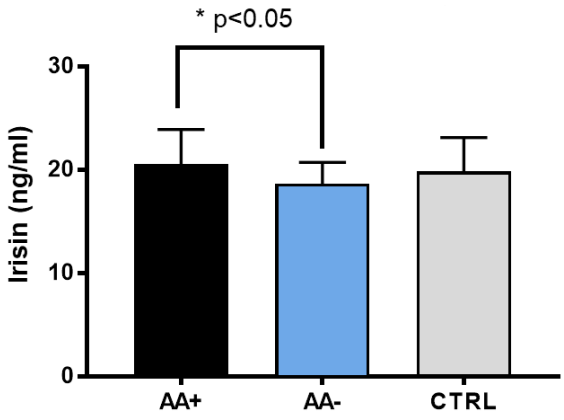

Figure 13: Irisin serum levels in AA+ and AA- patients as well as healthy controls. Irisin is modestly increased by 10% in AA+ patients compared to AA- ones (*p<0.05, two-tailed Student's t-test, mean ± SD).

Surprisingly, we did not find any correlation between irisin and BDNF serum concentrations, even though the first one has been shown to influence the production of this neurotrophin but, on the other hand, we observed a direct correlation of irisin serum levels with the duration of AA symptoms ($r=0.74$, $p<0.05$) (**Fig. 14**).

Following these general analyses of the TSPO/DBI peripheral system, we investigated the putative role of two different well-known polymorphisms in affecting some of its markers.

➢ Influence of the TSPO polymorphism rs6971

This polymorphism, identified as rs6971, is located in the TSPO gene and is an Ala147Thr substitution, which is found in the Caucasian population with these allelic frequencies: 49% Ala/Ala (G/G), 42% Ala/Thr (G/A) and 9% Thr/Thr (A/A).

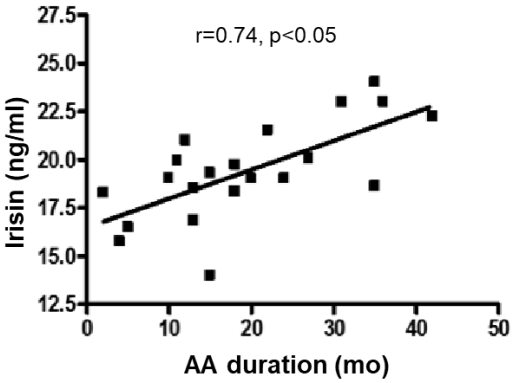

Figure 14: Direct correlation between irisin serum levels and the duration of AA symptom in AA+ patients. Irisin lower concentrations are found in subjects which have been manifesting AA disturbances for shorter times (*p<0.05).

These proportions were maintained in our AD population (**Fig. 15**) but we did not find any significant difference among the allelic subgroups as regards TSPO gene expression in PBMC (**Fig. 16**), NPI-12 scores (**Fig. 17**) or other markers related to the TSPO/DBI peripheral system (data not shown).

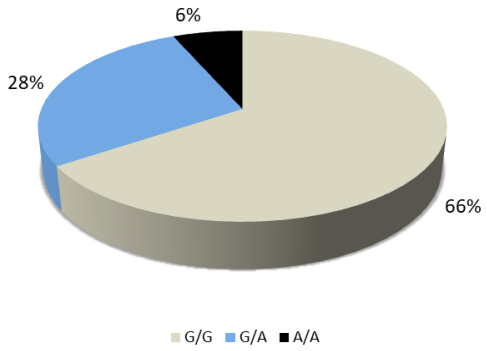

Figure 15: Distribution of the rs6971 TSPO polymorphism in our AD patients. The allelic frequencies reflect the ones described in literature.

Diversely, we observed a slight increase in DBI serum levels in the heterozygous subjects compared to the alanine homozygous ones (p<0.01) (**Fig. 18**).

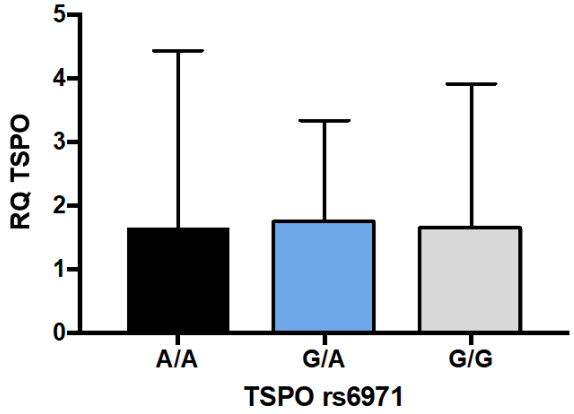

Figure 16: TSPO gene expression in PBMC of AD patients sorted by TSPO rs6971 polymorphism. TSPO gene expression is unchanged among the groups. Relative Quantification (RQ) of TSPO mRNA is calculated as ratio to β-actin (one-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD).

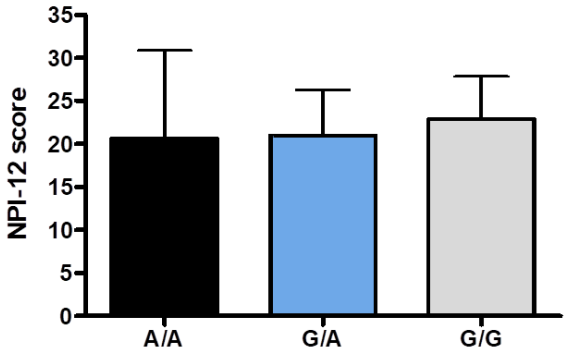

Figure 17: NPI-12 score in AD patients sorted by TSPO rs6971 polymorphism. NPI-12 score is unchanged among the groups (one-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD).

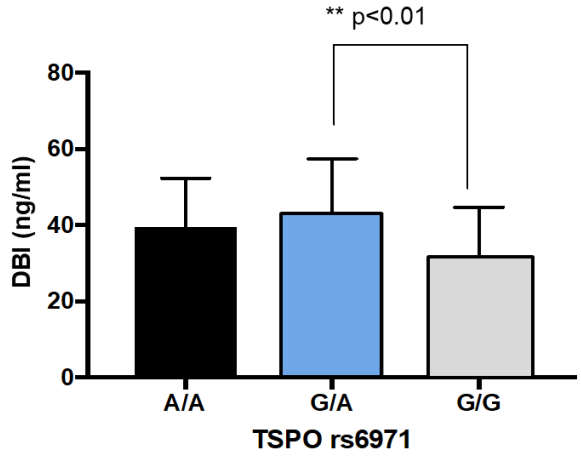

Figure 18: DBI serum levels in AD patients sorted by TSPO rs6971 polymorphism. DBI is mildly increased in G/A patients compared to G/G (**p<0.01, one-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD).

➢ Influence of the BDNF polymorphism rs6265

This polymorphism, recognized as rs6265, is situated in the BDNF gene and is a Val66Met substitution, which is observed in the Caucasian population with these allelic frequencies: 65% Val/Val (G/G), 32% Val/Met (G/A) and 3% Met/Met (A/A).

As it was for rs6971, the genotypes of our AD population reflected this distribution (**Fig. 19**) but, again, we did not identify any significant difference among the subgroups neither in serum BDNF levels (**Fig. 20**) nor in MMSE or NPI-12 scores.

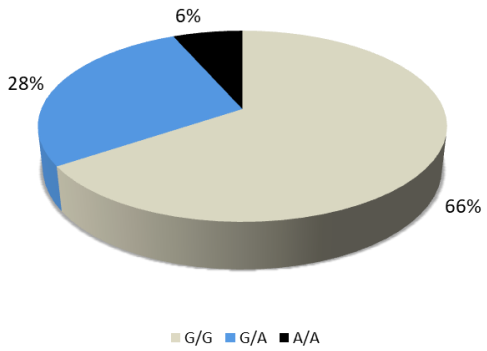

Figure 19: Distribution of the rs6265 BDNF polymorphism in our AD patients. The allelic frequencies reflect the ones described in literature.

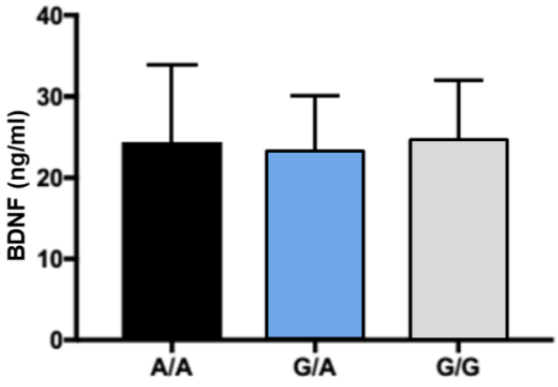

Figure 20: BDNF serum levels in AD patients sorted by BDNF rs6265 polymorphism. BDNF is unchanged among the subgroups (one-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD).

7.2 Mild Cognitive Impairment

➢ Recruited population

To verify if the increase in serum DBI levels that we observed in AD patients is specific for this pathology, we also recruited n=33 subjects classified in a *prodromal AD* stage, since they display an amnesic mild cognitive impairment (aMCI) together with neurodegeneration (confirmed by positive brain MRI and 18F-FDG PET). Not only they were characterized for different parameters (**Table 3**) but also they underwent a Flutemetamol-PET, in order to distinguish between amyloid positive (Amy+) and amyloid negative (Amy-) individuals, depending on the presence of amyloid deposits.

	MCI (n=33)
Sex (% M)	60%
Age (ys)	71 ± 6 (55-82)
Level of education (ys)	10 ± 4.7 (2-18)
MMSE (score)	26 ± 12.4 (24-29)
AChEI (n, %)	0%
Memantine (n, %)	0%
Amyloid PET (% positive)	70%
ApoE genotype	N/A

Table 3: Complete list of the clinical and demographic information about aMCI recruited population. The Flutemetamol-PET is positive in 23 subjects (70%) (mean ± SD) (N/A: not applicable; range intervals in brackets).

➢ DBI serum levels

We measured DBI serum concentration in our aMCI subjects and we compared these data with the ones from our initial cohorts of AD patients and healthy controls. Interestingly, the aMCI population showed a significant 52% increase (p=0.0192) and a significant 30% decrease (p=0.0003) in DBI serum levels compared to CTRL and AD groups, respectively (**Fig. 21**).

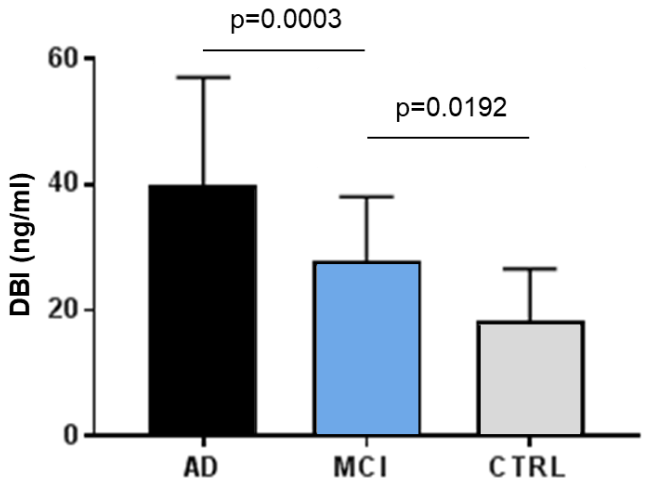

Figure 21: DBI serum levels in AD patients, aMCI subjects and healthy controls. DBI serum concentration is increased by 52% in aMCI compared to CTRL and decreased by 30% in aMCI compared to AD (***p<0.0001, one-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD).

Moreover, comparing Amy+ and Amy- subjects, we did not find any significant change in DBI serum levels (**Fig. 22**) as well as in other clinical-demographic parameters (data not shown).

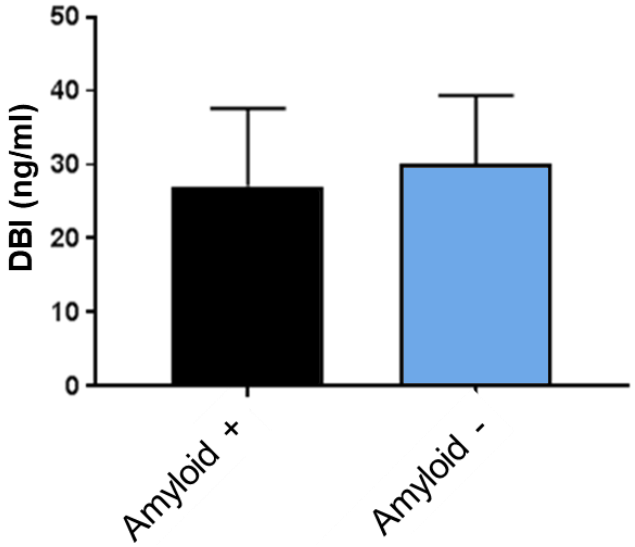

Figure 22: DBI serum levels in Amy+ (n=23) and Amy- (n=10) subjects. DBI serum concentration is unchanged between the two groups (p=0.46, two-tailed Student's t-test, mean ± SD).

7.3 Non-Determined Cognitive Impairment

➢ Recruited population

Beyond AD and aMCI population, we recruited a third cohort of n=38 patients displaying cognitive impairment, which could not be classified directly as AD subjects since they showed heterogeneous aspects in both clinical tests and imaging exams, ascribable to different types of dementia. Thus, after the first characterization, which is listed in **Table 4**, they underwent lumbar puncture to analyse their CSF profile of AD core markers Aβ, t-tau and p-tau.

	NDCI (n=38)
Sex (% M)	47%
Age (ys)	71.4 ± 9 (51-83)
Level of education (ys)	6.1 ± 2.8 (3-17)
Disease duration (mo)	35.8 ± 22.43 (1-93)
MMSE (score)	21.0 ± 5.5 (10-28)
MMSE>26	17%
CDR 1 / 2 / 3 (%)	47% / 36% / 0%
AChEI (n, %)	31%
Memantine (n, %)	13%

Table 4: Complete list of the demographic and clinical information about NDCI recruited population. The MMSE was above the threshold for cognitive impairment in 17% of the subjects (mean ± SD) (range intervals in brackets).

The typical AD profile is distinguished by Aβ<500 pg/ml and t-tau>300 pg/ml, thus we used the same cut-off to discriminate among our NDCI population. According to the CSF concentration of these indicators, 15 subjects were considered non-AD (Aβ>500 pg/ml), 17 were confirmed AD (Aβ<500 pg/ml and t-tau>300 pg/ml) and 6 were classified as suspect-AD (Aβ<500 pg/ml and t-tau<300 pg/ml). These subpopulations were screened for different parameters (**Table 5**) but these factors did not show significant changes among the subgroups (data not shown).

	AD (n=17)	Suspect AD (n=6)	Not AD (n=15)
Sex (% M)	40%	33%	60%
Age (ys)	71 ± 10.1 (51-83)	71 ± 7.7 (62-82)	72 ± 8.7 (59-82)
Level of education (ys)	8.6 ± 3.8 (5-17)	5.7 ± 2 (4-8)	10.7 ± 5.3 (3-16)
Disease duration (mo)	37.6 ± 27.2 (1-93)	25.5 ± 16.5 (9-45)	36.5 ± 17.5 (9-69)
MMSE (score)	19 ± 5.9 (10-27)	21 ± 4.2 (16-26)	23 ± 5 (14-28)

Table 5: Complete list of the demographic and clinical information about AD, suspect-AD and non-AD subjects in the initial NDCI population. The small differences in these parameters among the groups were not statistically significant (one-way ANOVA, mean ± SD) (range intervals in brackets).

➢ DBI serum and CSF levels

As we did for our earliest cohort of AD patients and for aMCI subjects, we measured DBI serum levels in these NDCI subpopulations and we compared them with our initial healthy controls. Interestingly, DBI serum concentration was increased by 61% in AD individuals compared to non-AD ones (p=0.045), whereas the apparent increase in suspect-AD subjects compared to non-AD ones was not significant (**Fig. 23**).

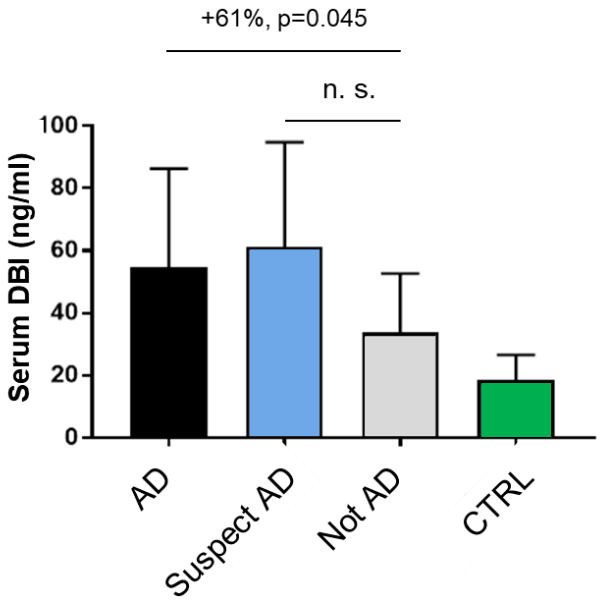

Figure 23: DBI serum levels in AD (n=17), suspect-AD (n=6), non-AD (n=15) subjects and healthy controls. DBI serum concentration is higher in AD than in non-AD (***p<0.0001, one-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD).

Considering the same subgroups, we also compared the DBI CSF levels among them but we did not observe any significant difference (**Fig. 24**).

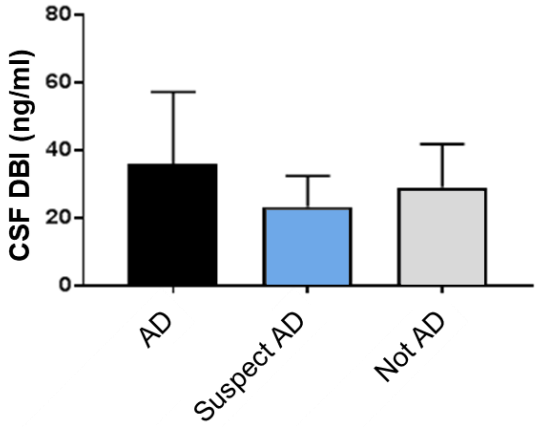

Figure 24: DBI CSF levels in AD (n=17), suspect-AD (n=6) and non-AD (n=15) subjects. DBI CSF concentration is unchanged among the subgroups (p=0.25, one-way ANOVA, mean ± SD).

On the contrary, comparing DBI concentrations between serum and CSF in the whole NDCI population, we observed a significant 52% increase of DBI levels in the serum compartment (**Fig. 25**) and similar results were detected considering the NDCI subgroups separately (data not shown).

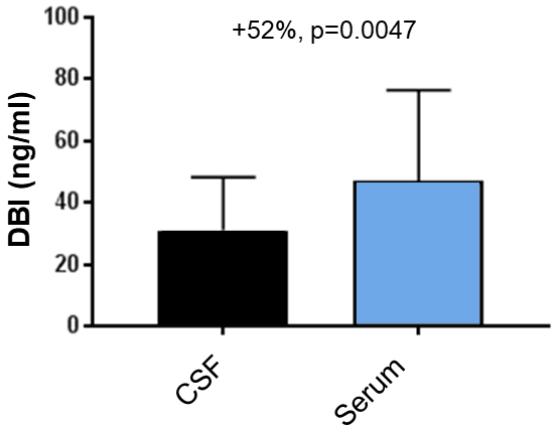

Figure 25: DBI CSF and serum levels in NDCI recruited population. DBI concentration is higher in the serum than in CSF (p=0.0047, two-tailed Student's t-test, mean ± SD).

Interestingly, analysing the whole NDCI population, we also found a direct correlation between DBI CSF concentration and its levels in serum (r=0.45, p=0.0052) (**Fig. 26**).

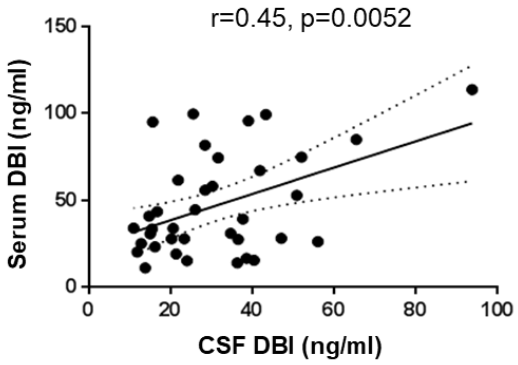

Figure 26: Direct correlation between DBI CSF levels and DBI serum levels in NDCI patients. DBI CSF concentration is significantly correlated to DBI serum one (p=0.0052).

➢ DBI CSF levels and AD core-markers

In order to highlight a possible relationship between DBI CSF levels and AD core-markers, we analysed its correlations with Aβ, t-tau and p-tau. Whether we did not find any with the first marker (data not shown), we identified an interesting correlation of DBI CSF concentration with both t-tau (r=0.63, p<0.0001) (**Fig. 27A**) and p-tau (r=0.46, p=0.0036) (**Fig. 27B**).

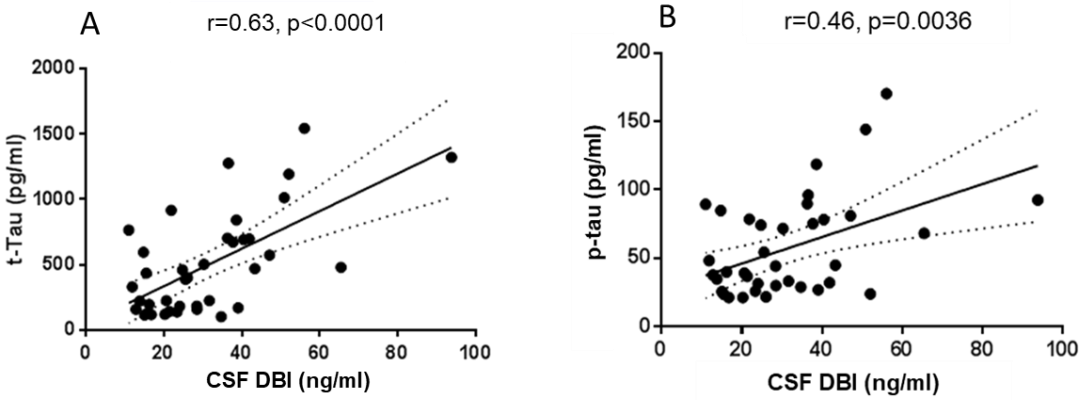

Figure 27: (A) Direct correlation between DBI and t-tau CSF levels in NDCI patients (*p<0.0001). (B) Direct correlation between DBI and p-tau CSF levels in NDCI patients (*p<0.05).**

➢ DBI serum and CSF levels after AA dichotomization

Among the AD cohort identified in our NDCI population, we selected 12 patients which were administered clinical tests for cognitive impairment and BPSD measurement, such as MMSE, NPI-

12 and CBI. Moreover, we dichotomized them according to the presence of the AA symptom and we calculated the AA score (**Table 6**). We did not find any difference between the two groups as regards the collected parameters except for NPI-12, which was higher in AA+ patients than in AA- ones (34.0 ± 10.7 vs. 15.6 ± 12.6, p=0.04).

We analysed DBI levels in AA+ and AA- subjects, considering both serum and CSF compartments. However, we did not find any difference in DBI serum or CSF concentration between the two groups. Similarly, we did not observe any significant change comparing DBI serum levels to its CSF ones within both AA+ and AA- cohorts (**Fig. 28**).

	AA (n=12)	AD AA+ (n=6)	AD AA- (n=6)
Sex (% M)	25%	33%	17%
Age (ys)	70.3 ± 9.5 (51-82)	72 ± 9 (59-82)	68.8 ± 10.4 (51-79)
Level of education (ys)	7.7 ± 3.9 (4-17)	6.8 ± 3.5 (4-13)	8.5 ± 4.4 (5-17)
Disease duration (mo)	25.2 ± 11.1 (9-44)	19.2 ± 7.3 (9-27)	31.2 ± 11.5 (11-44)
MMSE (score)	23.1 ± 3.8 (15-27)	22.8 ± 3 (19-27)	23.3 ± 4.8 (15-27)
NPI-12 (score)	24.8 ± 14.7 (2-48)	34 ± 10.7 (21-48)	15.6 ± 12.6 (2-33)
AA (score)	1.8 ± 3.4 (0-12)	3.7 ± 4.3 (1-12)	N/A
CBI (score)	17.7 ± 13.4 (1-36)	21.7 ± 12 (7-35)	13.8 ± 14.6 (1-36)

Table 6: Complete list of the demographic and clinical information about 12 AD subjects in the initial NDCI population. They were characterized for behavioural disturbances and divided in AA+ and AA- patients, according to the presence of the AA symptom. Only the NPI-12 was higher in the AA+ subjects that in the AA- ones (p=0.04, Student's t-test, mean ± SD) (range intervals in brackets).

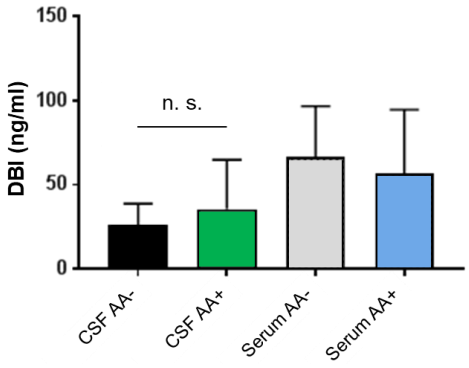

Figure 28: DBI CSF and serum levels in 12 AD subjects of the recruited NDCI population dichotomized for AA symptom. DBI concentration is unchanged in both CSF and serum between AA+ and AA- subject. DBI levels are unchanged also between serum and CSF within both AA+ and AA- populations (two-tailed Student's t-test, mean ± SD).

7.4 Delirium

➢ Recruited population

In addition to our main populations, we also recruited n=6 patients that developed delirium during their hospitalization, which was ascribable to different reasons, including cerebrovascular accidents and brain tumours. Delirium was suspected based on the 4AT score (a score ≥4 is indicative of possible delirium ± cognitive impairment). Patients characteristics are included in **Table 7**.

	Delirium (n=6)
Sex (% M)	3 (50%)
Age (ys)	73.7 ± 9.7 (54-79)*
4AT score°	10.9 ± 2.6 (6-12)
Ammonia (µM)[#, a]	47.0 ± 13.3 (33-69)
Sodium[#, b]	138.5 ± 5.9 (125-144)
Potassium[#, c]	4.2 ± 0.4 (3.7-4.8)
Hyperkinetic form (%)	5 (83.3%)
Previous history of cognitive impairment/dementia (%)	3 (50%)

Table 7: Complete list of the demographic and clinical information about 6 delirium subjects. Delirium is suspected based on the 4AT score. (*median: 77.5 ys; °a score ≥4 is indicative of possible delirium ± cognitive impairment; #serum determinations; [a]all values within reference range (40-80 µg/dL); [b]only one patient below reference range values (136-145 mEq/L); [c]all values within reference range (3.5-5.1 mEq/L) (range intervals in brackets).

➢ DBI serum levels

As regards DBI serum concentration in delirium patients, this peptide was 3-fold higher than in healthy controls and increased by +80% compared to AD subjects (**Fig. 29**).

➢ Pro-inflammatory cytokines measurement

Since delirium is characterized by a strong systemic inflammatory response, we measured the serum concentration of both pro-inflammatory cytokines TNF-α and IL-6 not only in our delirium cohort but also in a subgroup of n=24 AD patients and n=8 healthy controls.

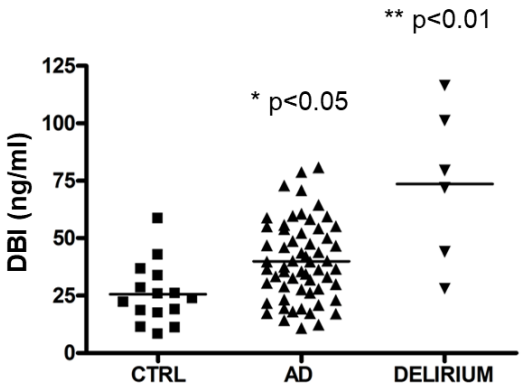

Figure 29: DBI serum levels in delirium subjects, AD patients and healthy controls. DBI concentration in delirium subjects is 3-fold higher than in healthy controls (*p<0.05) and is increased by 80% compared to AD patients (**p<0.01) (one-way ANOVA, followed by Tukey's multiple comparison test, mean ± SD).

According to our results, TNF-α did not show any change among the three groups, neither after its reassessment considering clinical and demographic variables (**Fig. 30A**). On the contrary, IL-6 was significantly increased in delirium patients compared to both AD subject and healthy controls (5-fold increase, p<0.001) (**Fig. 30B**).

These evidences were not modified by clinical or demographic data and, in particular, behavioural data failed to segregate with cytokine serum levels.

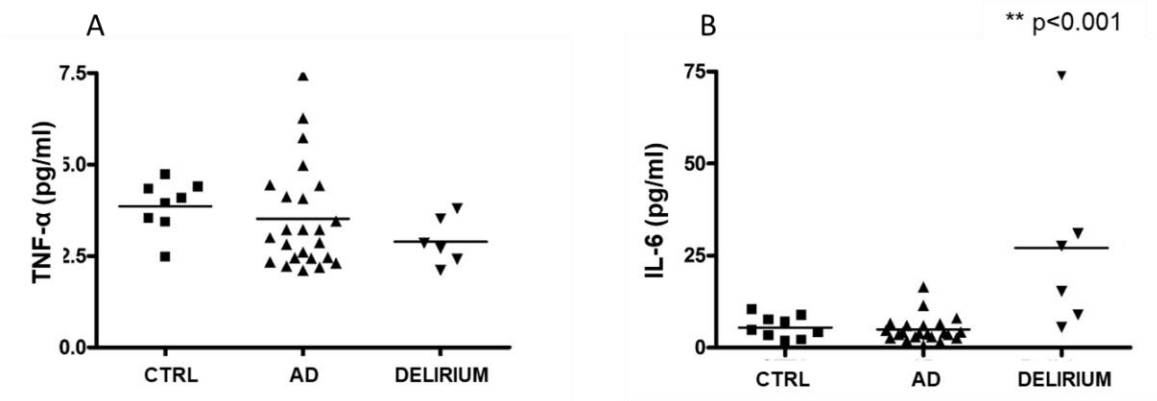

Figure 30: Cytokine serum levels in delirium subjects, AD patients (n=24) and healthy controls (n=8). (A) TNF-α serum levels are unchanged among the groups (p=0.34)**. (B)** IL-6 serum levels are 5-fold higher in delirium patients than in AD patients as well as healthy controls (**p<0.001). Even discarding the upper outlier in the delirium group the result significance is maintained (One-way ANOVA, followed by Tukey's multiple comparison test, mean ± SD).

7.5 Monocytes Chemotaxis

Beyond being involved in neurosteroids' production, TSPO has been demonstrated to regulate monocytes migration. Thus, we evaluated this process in response to either an oligomeric Aβ stimulus or a Ro5-4864 inhibition, using two different cellular types as well as chemotaxis assays.

➢ THP-1 cellular migration in μ-slide chemotaxis chambers

THP-1 cells, deriving from acute myeloid leukaemia and resembling primary monocytes from several point of views, represented a perfect cellular model to perform preliminary studies on putative Aβ-induced cellular migration using μ-slide chemotaxis chambers. This tool allowed us to observe THP-1 motility in time-lapse microscopy and to measure quantitative parameters such as direction, velocity and Forward Migration Index (FMI). This factor is defined as the parallel component of the chemotactic gradient and the more its values differ from 0, the wider is cellular migration.

The cells were administered either Aβ 125 nM or MCP-1 10 ng/ml, which was chosen as a positive control, being the most potent chemoattractant for monocytes.

As shown by **Fig. 31**, both substances induced a 100% increase in THP-1 cells chemotaxis compared to the basal condition (FMI: Aβ 0.36 ± 0.02 vs. MCP-1 0.35 ± 0.04 vs. basal 0.18 ± 0.04; $p<0.01$ Aβ vs. basal; $p<0.01$ MCP-1 vs. basal).

➢ Healthy controls monocytes migration in Boyden chambers

In parallel with THP-1 cells chemotaxis, we tested the effect of Aβ and MCP-1 in inducing the same process in human monocytes from elderly healthy controls. For this purpose, we used Boyden chambers that provided us the possibility to calculate the Chemotactic Index, which is recognized as a measure of cellular migration. We stimulated the cells with three different concentrations of Aβ oligomers, in order to highlight any potential dose-dependent difference in monocytes motility.

Comparing the basal condition with Aβ-treated cells, we did not detect any change in their migration for Aβ 125 or 1.25 nM, whereas cellular chemotaxis increased by 150% following the administration of Aβ 125 pM ($p<0.05$), a concentration which is placed within the detectable range

in human biological fluids. However, this effect was very mild in comparison with the one exerted by MCP-1, which boosted monocytes migration by 900% (p<0.01) (**Fig. 32**).

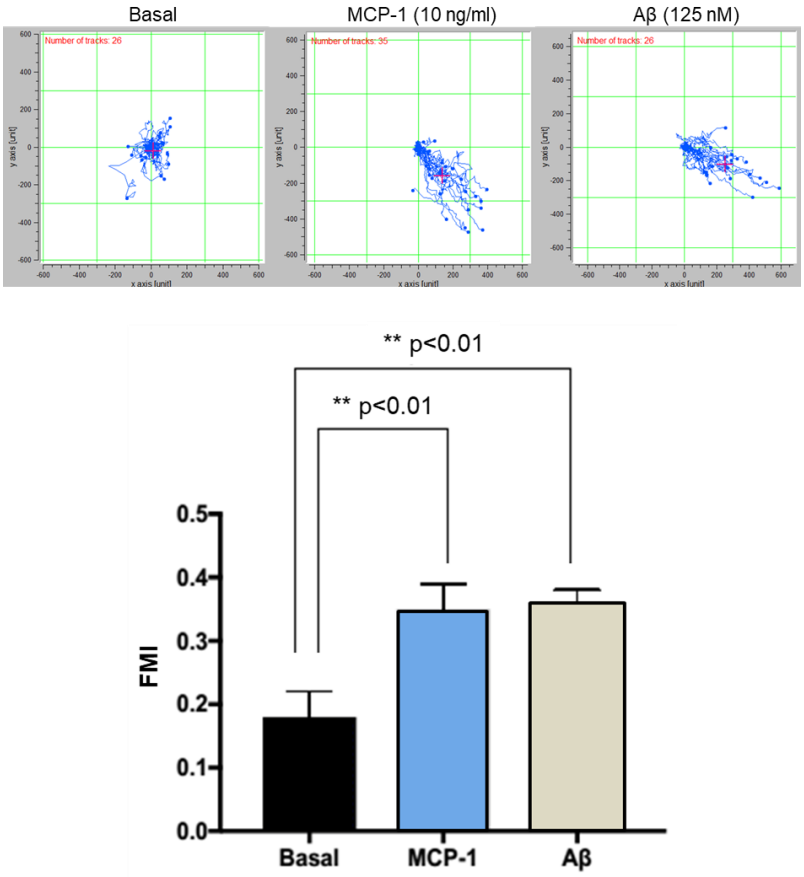

Figure 31: Forward Migration Index of THP-1 cells in µ-slide chemotaxis chambers. THP-1 cells migration increases, in comparison with the basal, after stimulation with MCP-1 10 ng/ml (**p<0.01) as well as Aβ 125 nM (**p<0.01). (One-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD).

In order to verify if the Aβ-induced migration that we observed was TSPO-dependent, we treated the same cells with Ro5-4864, which is known to inhibit this receptor. We tested this compound in different concentrations (10 µM, 25 µM and 50 µM), alone or in combination with Aβ. Besides confirming once again the strong chemoattractant effect of Aβ 125 pM, the results showed a strong inhibitory effect of Ro5-4864 on Aβ-induced chemotaxis. Indeed, when this synthetic molecule was present in the medium together with Aβ, the cellular migration was significantly lower than the one boosted by Aβ alone, especially at Ro5-4864 concentrations of 25 and 50 µM (Aβ: 4.41 ± 2.45 vs. Ro5 25 µM: 1.15 ± 0.35, p<0.05; Aβ: 4.41 ± 2.45 vs. Ro5 50 µM: 0.95 ± 0.85,

p<0.05) (**Fig. 33**). As expected, monocytes stimulation with Ro5-4864 alone did not exhibit any significant difference compared to the basal condition.

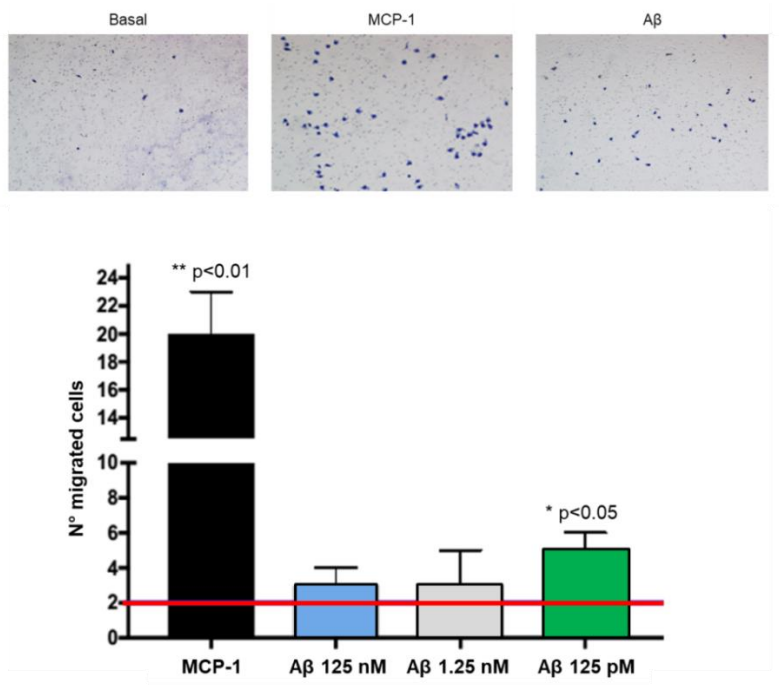

Figure 32: Elderly healthy controls monocytes chemotaxis in Boyden chambers. Aβ 125 nM as well 1.25 nM concentrations don't have any effect in inducing monocytes chemotaxis in comparison to the basal condition (red line), whereas Aβ 125 pM increases their migration rate by 150% (*p<0.05). Nevertheless, MCP-1 has the strongest chemoattractant effect and stimulates monocytes chemotaxis by 900% compared to the basal condition (**p<0.01). (One-way ANOVA, followed by Bonferroni's post-hoc test, mean ± SD) (representative Aβ 125 pM staining).

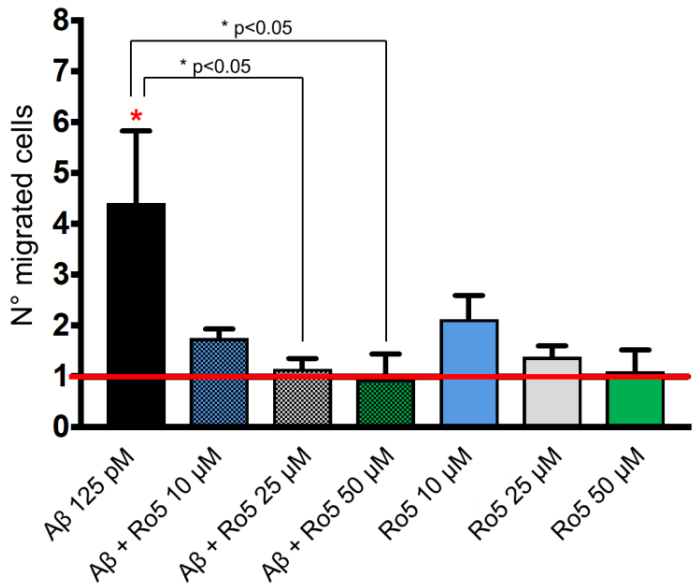

Figure 33: Elderly healthy controls monocytes chemotaxis in Boyden chambers. Aβ 125 pM confirms a strong effect in inducing monocytes chemotaxis in comparison to the basal condition (red line) (*p<0.05), whereas Ro5-4864 at medium and high concentrations significantly reduces monocytes migration when administered in combination with Aβ (Ro5 25 and 50 µM: *p<0.05, one-way ANOVA, followed by Tukey's post-hoc test, mean ± SD).

➢ Monocytes migration in AD patients

To highlight any potential difference in monocytes chemotaxis between healthy controls and AD patients, we stimulated this process in two small subgroups (n=5) of both populations using Aβ 125 pM as chemoattractant. We observed that the migration rate of the monocytes isolated from AD patients was 100% higher than the one from healthy controls cells (4.4 ± 0.6 vs. 2.0 ± 0.4, p<0.01) (**Fig. 34**).

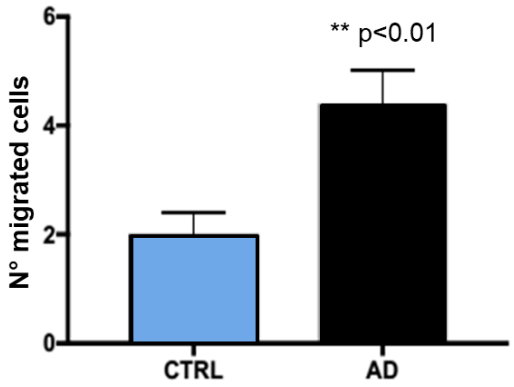

Figure 34: Aβ-induced chemotaxis of two subgroups (n=5) of healthy controls and AD patients. Aβ 125 pM induces a stronger chemotactic response (indicated as number of migrated cells) in monocytes from AD patients than in ones from healthy controls (**p<0.01) (Student's t-test, mean ± SD).

Chapter 8

Results (part 2)

8.1 Neutrophils Infiltration

➢ Experimental design

We performed immunohistochemical analyses on mouse brain slices that were kindly provided by the research group headed by Prof. Michael T. Heneka at the University of Bonn Medical Centre (Bonn, Germany). The samples belonged to 4 genetic backgrounds (WT, *Nlrp3*$^{-/-}$, APP/PS1, APP/PS1/*Nlrp3*$^{-/-}$), 2 ages (5 and 15 months old) and 3 different treatments (untreated (CTRL), 2 days (2d) and 10 days (10d) after a single LPS injection), according to the diagram in **Fig. 35**, for a total of 24 different conditions.

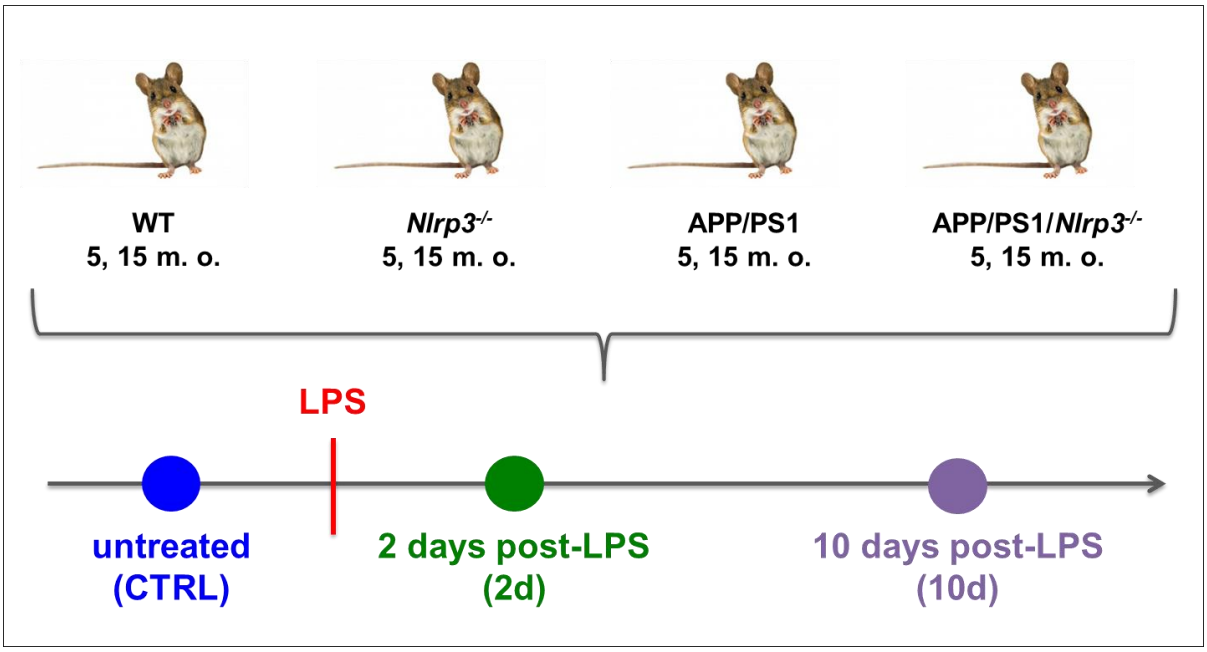

Figure 35: Diagram reporting the experimental design to perform immunohistochemistry on mouse brain slices. The samples derived from WT, *Nlrp3*$^{-/-}$, APP/PS1 and APP/PS1/*Nlrp3*$^{-/-}$ mice, which were considered in their young (5 m. o.) as well as old (15 m. o.) age. For each genetic background and age, three different conditions were analysed: untreated (CTRL), 2 and 10 days after LPS injection (2d and 10d, respectively).

To identify the putative presence of activated neutrophils, we stained our brain slices with MPO together with NE, which are both recognized as markers of NETs. In addition, we labelled the tissue with methoxy-X04 and DAPI, in order to visualize amyloid deposits and cell nuclei, respectively. With the aim to highlight any Aβ-dependent effect in cellular infiltration, we analysed our brain slices distinguishing between plaque-associated and plaque-free areas.

➢ Plaque-free areas analysis

Considering plaque-free areas, we did not detect any positive cell in brain slices, regardless of mice genotype, age and treatment (**Fig. 36**, pictures from WT and *Nlrp3*$^{-/-}$ mice not shown).

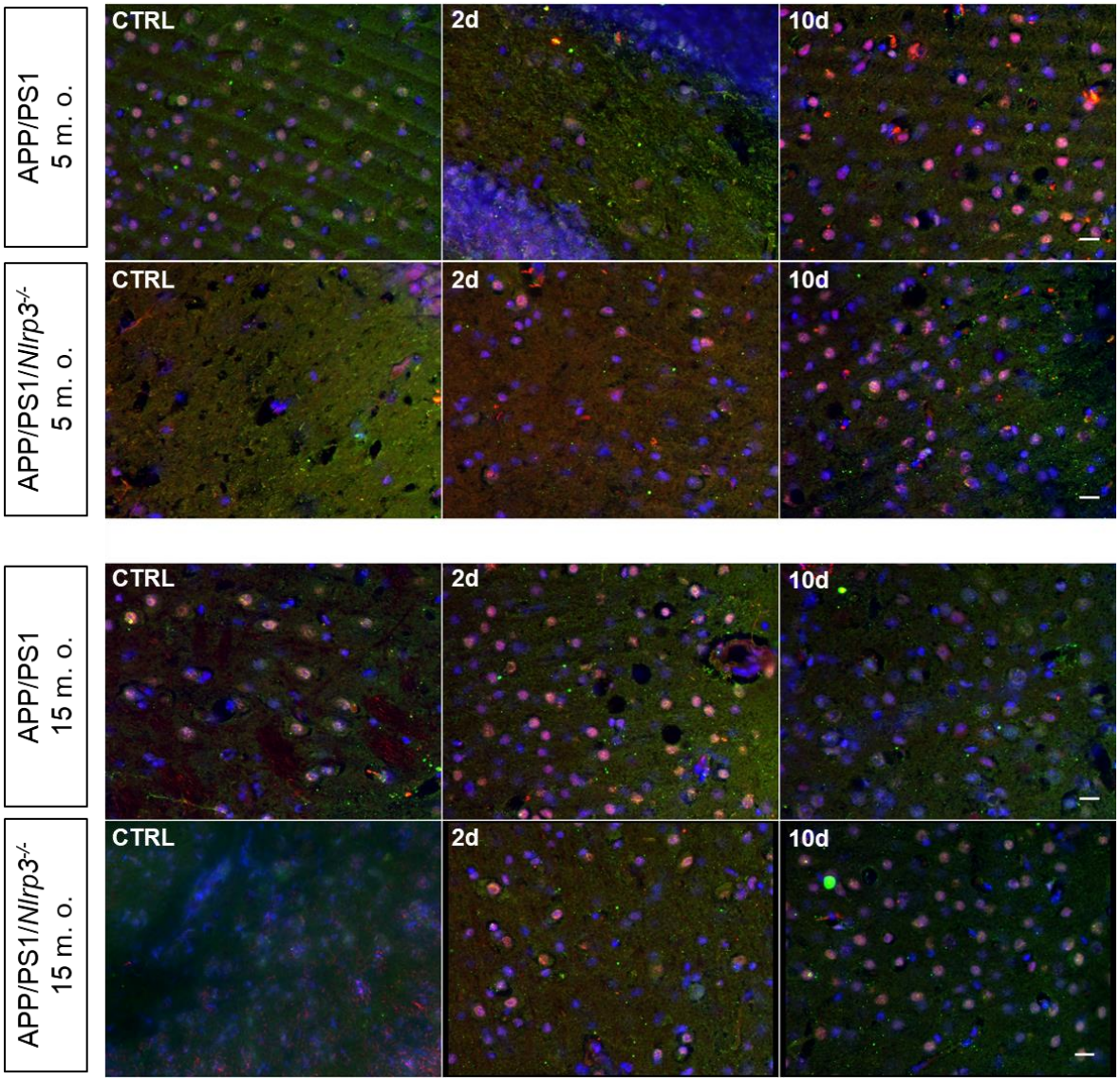

Figure 36: Representative fluorescence microscopy pictures of plaque-free areas in brain slices from APP/PS1 and APP/PS1/*Nlrp3*$^{-/-}$ mice belonging to different age and treatment groups. There are no MPO^{+}/NE^{+} cells in plaque-free areas, independently from animal genotype, age or treatment. Nuclei are visualized using the fluorescent nuclear dye DAPI (blue). Scale bar: 20 µm.

➢ Plaque-associated areas analysis

Given that amyloid plaques represent one of the main hallmarks of AD pathology, we did not detect them neither in WT nor in *Nlrp3*$^{-/-}$ mice brain slices. Thus, we excluded these samples from further analyses. On the contrary, considering the brains of APP/PS1 as well as APP/PS1/*Nlrp3*$^{-/-}$ animals, not only we observed MPO and NE labelling (**Fig. 37, 38A**) but we also verified that these markers were co-localizing within the tissue (**Fig. 38B**). Moreover, these MPO^{+}/NE^{+} cells were mainly identified in close proximity to Aβ deposits.

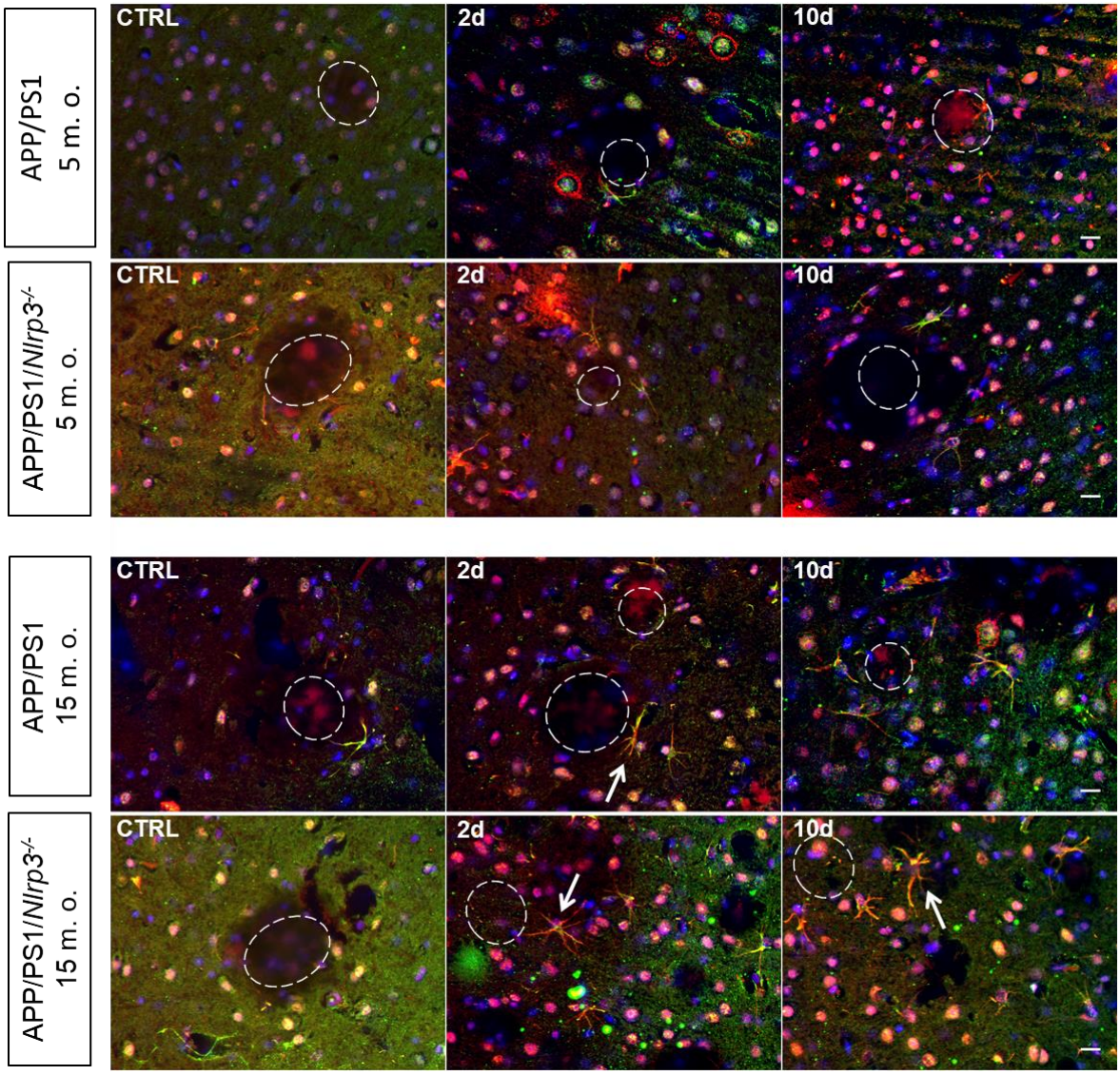

Figure 37: Representative fluorescence microscopy pictures of plaque-associated areas in brain slices from APP/PS1 and APP/PS1/*Nlrp3*$^{-/-}$ mice belonging to different age and treatment groups. MPO^{+} (green) and NE^{+} (red) signals are co-localizing in all the samples and the cells labelled by these two markers (see representative white arrows) are highly concentrated around amyloid plaques (see representative dotted circles). Nuclei and amyloid plaques are visualized using the fluorescent nuclear dye DAPI (blue) and the chemical Mx04 (blue but more visible in the red channel because of the strong DAPI signal), respectively. Scale bar: 20 µm.

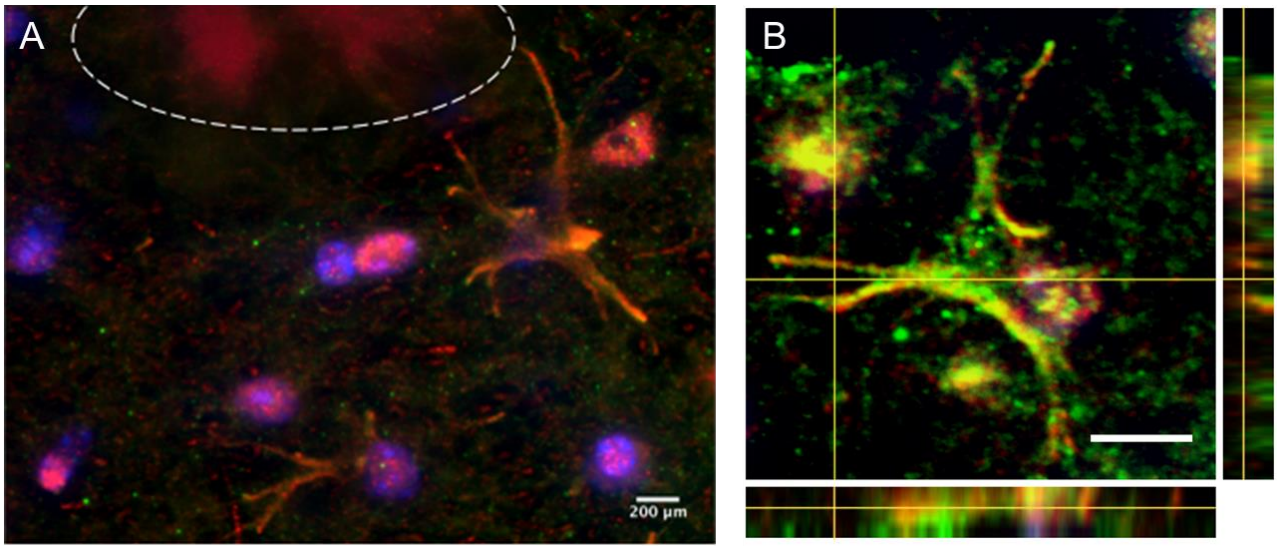

Figure 38: (A) Representative fluorescence microscopy picture of an MPO^+/NE^+ cell in a plaque-associated area in an APP/PS1 15 m. o. 2d mouse. MPO (green) and NE (red) co-staining. Nuclei and amyloid plaque are visualized using the fluorescent nuclear dye DAPI (blue) and the chemical Mx04 (blue but more visible in the red channel because of the strong DAPI signal), respectively. Scale bar: 200 µm. **(B): Representative orthogonal view of a fluorescence microscopy picture showing an MPO^+/NE^+ cell in a plaque-associated area in an APP/PS1 15 m. o. 2d mouse.** As shown by the orthogonal view, MPO and NE labellings are co-localizing in the same cell. Nucleus is visualized using the fluorescent nuclear dye DAPI (blue). Scale bar: 200 µm.

To highlight potential differences related to genetic background, age or treatment, we quantified the number of labelled neutrophils in the samples. In order to do that, we counted the positive cells in 4 optical fields (OF) per slice and we analysed 2 slices per brain, which means 8 optical fields per brain.

According to our manual count (**Fig. 39A**), the number of MPO^+/NE^+ cells infiltrating the brain of APP/PS1 mice increased in 15-month-old animals compared to the 5-month-old ones, in both 2-days and 10-days-post-LPS treated groups (0.25 ± 0.46 vs. 2.0 ± 1.20 cells/OF, p=0.0119 and 0.50 ± 0.76 vs. 3.38 ± 1.19 cells/OF, p<0.0001, respectively). Similarly, a higher cell number was detected in brain slices of APP/PS1/*Nlrp3*$^{-/-}$ mice 2 days as well as 10 days after LPS injection (1.75 ± 1.04 vs. 4.38 ± 1.51, p<0.0001 and 1.75 ± 1.98 vs. 5.0 ± 1.85, p<0.0001, respectively).

Interestingly, we observed an increase in the number of infiltrated neutrophils in 15-month-old APP/PS1 mice 2-days as well as 10-days post-LPS injection rather than in the untreated ones (0.75 ± 0.46 vs. 2.0 ± 1.20, p=0.0387 and 0.75 ± 0.46 vs. 3.38 ± 1.19, p<0.0001, respectively) (**Fig. 39B**). On the other hand, 15-month-old APP/PS1/*Nlrp3*$^{-/-}$ mice showed a higher number of cells per

optical field when comparing animals sacrificed 2 or 10 days post-LPS treatment to the untreated ones (0.50 ± 0.54 vs. 4.38 ± 1.51, p<0.0001 and 0.50 ± 0.54 vs. 5.0 ± 1.85, p<0.0001, respectively).

A similar effect was detected in APP/PS1/*Nlrp3*$^{-/-}$ 5 m. o. LPS-treated mice but its statistical significance was weaker than the ones reported above (data not shown).

In addition, considering old animals only, APP/PS1/*Nlrp3*$^{-/-}$ mice displayed a significant increase in the number of infiltrating neutrophils compared to the APP/PS1 ones, in both 2 or 10 days post-LPS treated groups (2.00 ± 1.20 vs. 4.38 ± 1.51, p<0.0003 and 3.36 ± 1.89 vs. 5.0 ± 1.85, p=0.0226, respectively) (**Fig. 39C**).

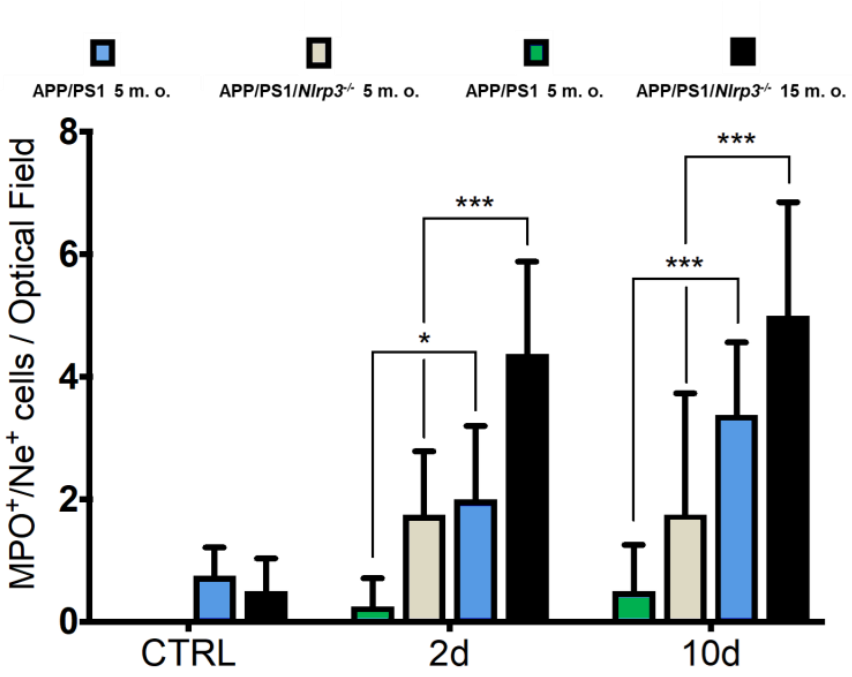

Figure 39: (A) Neutrophils manual count. The number of MPO^{+}/NE^{+} cells in each optical field increases in APP/PS1 as well as in APP/PS1/*Nlrp3*$^{-/-}$ older animals compared to the young ones in both LPS-treated groups (APP/PS1 2d: *p=0.0119; APP/PS1 10d: ***p<0.0001; APP/PS1/*Nlrp3*$^{-/-}$ 2d: ***p<0.0001; APP/PS1/*Nlrp3*$^{-/-}$ 10d: ***p<0.0001).(Two-way ANOVA, followed by Tukey's multiple comparisons test, mean ± SD).

Since positive cells to our labelling were found in close proximity to amyloid plaques only, we performed some additional experiments to exclude that MPO and NE could be expressed by microglia, which has already been demonstrated to cluster around Aβ deposits. To do that, we co-stained our samples using either MPO or NE with Iba1, which is considered the main microglial marker and we did not observe any co-localization between these proteins (**Fig. 40A-B**).

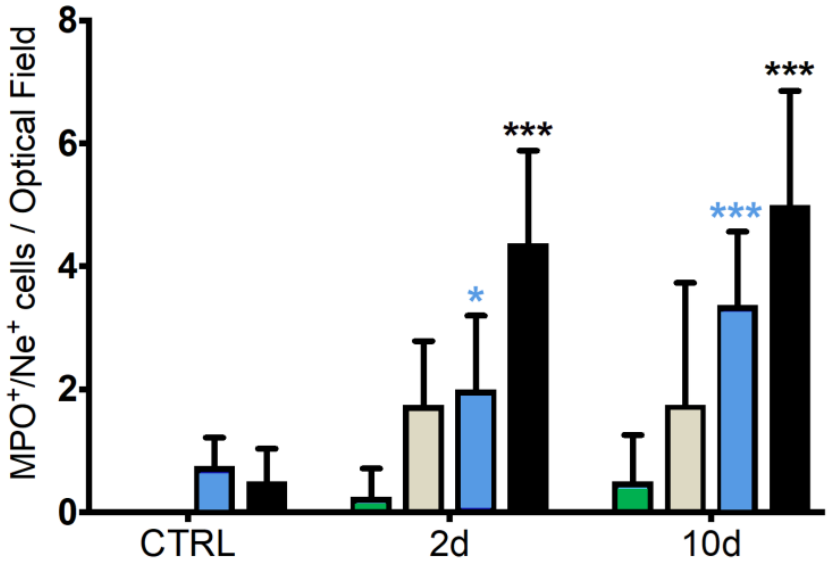

Figure 39: (B) Neutrophils manual count. The number of infiltrated neutrophils increases in LPS-treated mice compared to the untreated ones, regardless of their genetic background and this effect is much more evident in older animals than in younger ones (APP/PS1 15 m. o. 2d: *p=0.0387; APP/PS1 15 m. o. 10d: ***p<0.0001; APP/PS1/*Nlrp3*$^{-/-}$ 15 m. o. 2d: ***p<0.0001; APP/PS1/*Nlrp3*$^{-/-}$ 15 m. o. 10d: ***p<0.0001). (Two-way ANOVA, followed by Tukey's multiple comparisons test, mean ± SD).

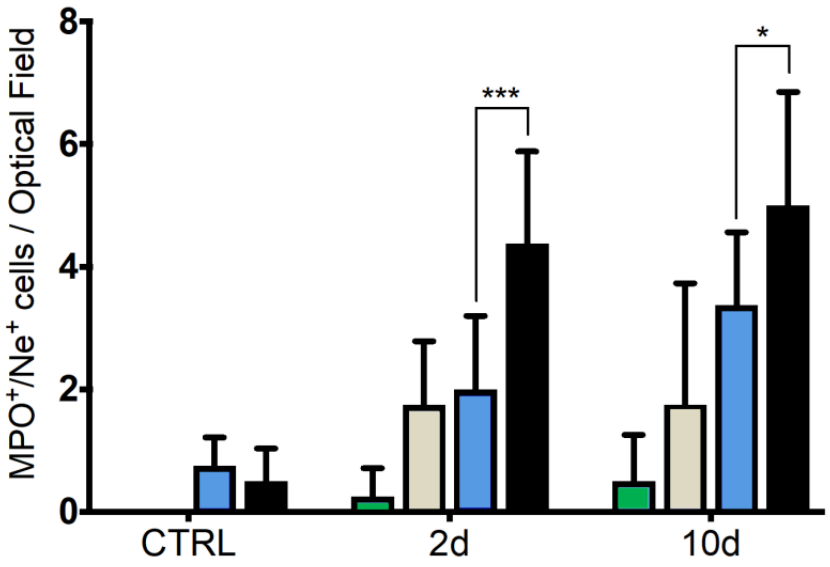

Figure 39: (C) Neutrophils manual count. In 15 m. o. animals, the number of infiltrated neutrophils increases in APP/PS1/*Nlrp3*$^{-/-}$ mice compared to the APP/PS1 ones, 2 or 10 days after LPS injection (2d: ***p=0.0003; 10d: *p=0.0226). (Two-way ANOVA, followed by Tukey's multiple comparisons test, mean ± SD).

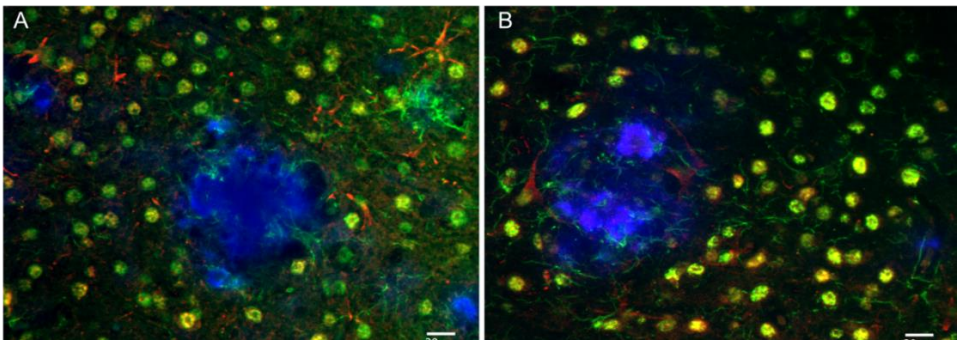

Figure 40: Representative fluorescence microscopy pictures of a plaque-associated area in an APPPS1 15 m. o. 2d mouse. Iba1^{+} (green) and MPO^{+} **(A)** or NE+ **(B)** (red) signals are not co-localizing around amyloid plaques. Amyloid plaques are visualized using the chemical Mx04 (blue). Scale bar: 20 µm.

➢ Technical issues

It has to be mentioned that we displayed an aspecific signal in all the samples we stained. This labelling appeared in form of nuclei, which were diffused in the whole tissue and we hypothesized that it could most likely depend on an interaction between Sudan Black and the detergent contained in the washing buffer, rather than on the MPO and NE antibodies. To verify this assumption, we stained several WT 5 m. o. CTRL brain slices for non-proliferating (Iba1^{+}) as well as proliferating (Ki-67^{+}) microglial cells and we observed the same aspecific signal (**Fig. 41A**). Further attempts to stain the same sample for microglia (Iba^{+}) and astrocytes (GFAP^{+}) without detergent were done and they were successful in removing the nuclei-like signal (**Fig. 41B**) but, even though this new protocol was effective, we could not perform again immunohistochemistry on the mice slices included in our experimental design because they were no longer available.

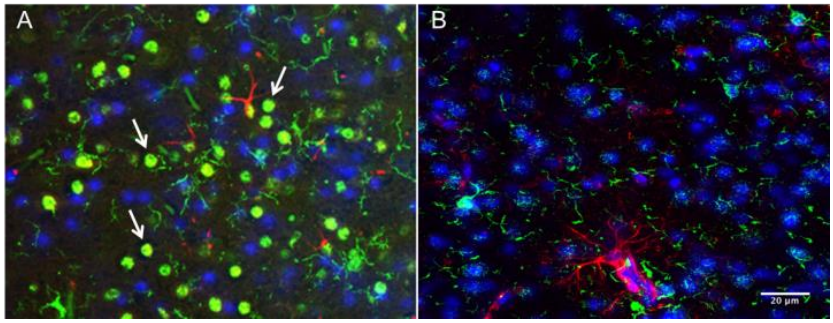

Figure 41: (A) Representative fluorescence microscopy picture of a WT 5 m. o. CTRL mouse brain slice. Aspecific nuclei (see white arrows) are diffused in the whole slice. Non-proliferating microglia is stained with Iba1 (green), whereas the proliferating one is labelled with Ki-67 (red). Nuclei are visualized using the fluorescent nuclear dye DAPI (blue). Scale bar: 20 µm. **(B) Representative fluorescence microscopy picture of a WT 5 m. o. CTRL mouse brain slice.** With the new protocol, the aspecific signal disappeared and microglia (green) as well as astrocytes (red) are clearly visible. Nuclei are visualized using the fluorescent nuclear dye DAPI (blue). Scale bar: 20 µm.

Chapter 9

Discussion

9.1 Discussion of the Study

This study was aimed to investigate some biological mechanisms potentially involved in the genesis of several behavioural disturbances often associated to Alzheimer's Disease. Moreover, it intended to examine the pathological contribution of peripheral inflammatory responses exerted by monocytes and neutrophils in boosting central neuroinflammation.

For our first purpose, we characterized behavioural anomalies in a cohort of subjects that were diagnosed AD according to their clinical as well as neuropsychological profile and we measured their behavioural manifestations by means of NPI-12, which is the most common caregiver-based scale employed to quantify BPSD. It has to be noticed that the individuals displaying a major expression of these symptoms might have been deleted from the sample due to the tight exclusion criteria but this choice, on the other hand, allowed us to better define the experimental groups and to avoid aspecific alterations of the measured parameters. However, the behavioural disturbances were extremely frequent in the recruited population, being present in more than 90% of AD subjects and this percentage paralleled the one reported by Corbett et al.[1053]. Apathy was confirmed to be the most recurrent manifestation, followed by depression and agitation/aggression. These data were in line with the ones reported in literature, except for depressive disturbances, which have been shown to have a 20% prevalence[1054] but that were much more common in our cohort. On the other hand, the distribution of BPSD in syndromic clusters revealed that apathetic manifestations were still the most represented, followed by affective and psychomotor ones. Their frequencies were congruous with the data reported by Spalletta et al.[1055], which grouped BPSD using the same clusters. Moreover, in their study as well as in ours, more than 80% of patients belonged to more than one cluster. As regards BPSD as a whole, the inverse correlation of NPI-12 score to the MMSE one, together with the direct correlation between NPI-12 score and disease duration, indicate that BPSD frequency as well as severity increase in parallel with cognitive impairment, as previously shown by Cerejeira et al.[989]. Also, the direct correlation between NPI-12 and CBI scores suggests that BPSD are essentially involved in generating caregiver stress burden.

However, it is worth to notice that behavioural anomalies are inhomogeneous and this feature is responsible for the first bias of our study. Indeed, the same NPI-12 scores could be associated to

completely different behavioural manifestations profiles. Thus, these disturbances are characterized by huge dissimilarities as concerns their clinical as well as therapeutic treatment.

For these reasons, we dichotomized our AD population according to the presence or absence of the agitation/aggression symptom, which is not only the most difficult to manage for the caregiver but also represents the main cause to demand for medical and pharmacological help. Also, displaying evident alterations, this anomaly is very easy to identify and facilitates the research for a link between BPSD and clinical or biological parameters.

Comparing AA+ subjects to the AA- ones, we did not find any significant difference in age, sex, level of education as well as disease duration between the two groups. On the contrary, we observed a lower MMSE score in AA+ patients, which was consistent with the hypothesis from Förstl and Kurz, stating that agitation/aggression symptom is typical of advanced dementia stages[1056].

Moreover, NPI-12 score was significantly higher in AA+ subjects than in AA- ones and this evidence might be explained suggesting that the agitation/aggression symptom is more frequently reported by the caregiver in comparison to other BPSD, because of its complicated features. In line with that, the CBI scores registered in caregivers of AA+ patients were much more higher than the ones recorded in caregivers dealing with non-aggressive subjects and this finding highlights the strong impact deriving from agitation/aggression in complicating AD patients nursing. This concept is strengthened by the observation that AA+ patients showed a more frequent use of antipsychotics and were more often assisted by caregivers external to their families, even though their cognitive impairment, determined by MMSE, was only mildly higher than the one detected in AA- subjects.

Furthermore, we examined the severity of the agitation/aggression symptom calculating the AA score, which represents a sub-scale of NPI-12. Thus, even though this measure is not very specific for the analysis of AA disturbances, it was helpful in this explorative study because it considered AA in relation to other BPSD rather than alone. Nevertheless, future studies might employ dedicated scales for agitation, such as the Cohen-Mansfield Agitation Inventory. Interestingly, we found an inverse correlation of the AA score with the years of education in agitated patients, although this second parameter did not show any significant difference between AA+ and AA- subjects. This finding suggests that a wider cognitive reserve, deriving from a longer education, might limit the severity of AA symptoms by stimulating inhibitory mechanisms, even though it is not protective against the appearance of these disturbances[1057]. This evidence underlines the

need to examine in details the role of cognitive reserve in modulating BPSD manifestations by means of specific tests like CRIq.

The ApoE genotype, despite influencing the risk for AD onset, did not display any involvement in modulating BPSD incidence in our diseased cohort.

Beyond characterizing behavioural anomalies from a clinical point of view, we also performed laboratory tests on biological samples collected right after NPI-12 administration and deriving from different diseased cohorts (AD, MCI, NDCI and delirium) as well as healthy controls, with the aim to investigate potential peripheral alterations in the TSPO/DBI system, which we hypothesized to be involved in BPSD genesis, in order to unravel a biomarker that could be useful to recognize either AD or BPSD.

The TSPO receptor, together with its endogenous ligand DBI, has been shown to be expressed in peripheral tissues[1058]. Moreover, it has been demonstrated that a bi-directional communication between systemic and CNS immunity exists[1059], raising the possibility to identify neuroinflammation-related biochemical changes not only in the brain but also in peripheral biological fluids. This opportunity offers a great advantage, since the procedure to obtain blood samples is minimally invasive and inexpensive.

The soluble mediator DBI was quantified in serum samples and it displayed increased levels in AD patients (+118%) compared to healthy controls. Also, in the AD cohort, the concentration of this peptide did not show any correlation with MMSE score or dementia severity, expressed by CDR score. As far as we know, there are no other studies in literature analysing DBI in AD patients serum.

The real peripheral function of the TSPO endogenous ligand has not been established yet and its role is still under debate. Its increased levels in AD patients might indicate that this peptide has a role in the pathology. On the other hand, the intact liver and kidney functions of the diseased subjects as well as their complete blood count with differential within the ranges provided by our analyses laboratory allowed us to exclude a DBI degradation deficiency, thus its higher concentration is reasonably ascribable to its increased synthesis. However, the source of this up-regulated process has still to be identified, since many different tissues are able to produce this peptide. Whether DBI increased serum concentration leads to the onset of pathological

disturbances or represents a compensatory mechanism to fight disease alterations has not been clarified to date but our evidence reflects a process occurring at a central level.

Indeed, Ferrarese et al. previously demonstrated the presence of higher DBI levels in the CSF of AD patients compared to healthy controls and they suggested that this increase might be correlated to cognitive decline[1039]. However, DBI functions in the CNS have not been defined completely but a few studies proposed that this peptide could be endowed with anxiogenic properties, since it acts as an inverse agonist of the $GABA_A$ receptor. On the other hand, it is reasonable to hypothesize that DBI is involved in neurosteroidogenesis, since this process is mediated by its receptor TSPO. Moreover, neurosteroids are direct modulators of the $GABA_A$ receptor and they promote the synthesis of neurotrophins such as BDNF, which are key mediators in the neurodegenerative process.

Thus, even though the real function of DBI is still largely unknown, its regulatory role on the GABAergic system has been already demonstrated and it might influence the genesis of BPSD in AD subjects.

However, some literature data underline that DBI CSF increased levels are found not only in AD patients but also in other neurodegenerative diseases displaying dementia traits, such as dementia-associated Parkinson's Disease[1039,1060]. Also, Ferrarese et al. found a reduced DBI concentration in CSF of patients suffering from Huntington chorea and hypothesized that this alteration could be due to GABAergic neurons degeneration. Thus, this peptide might not represent neither a specific biomarker nor a useful diagnostic tool to identify AD pathology, also given the wide overlap in its serum measurements between diseased subjects and healthy controls.

In order to examine the specificity of DBI as a marker of AD, we recruited other two diseased cohorts, represented by an MCI population as well as a group of patients displaying cognitive impairment, which has not been clearly classified yet (NDCI).

In MCI subjects, DBI serum concentration displayed values in-between healthy controls and AD patients, showing a 52% increase compared to the first population and a 30% decrease compared to the second one. On the other hand, we characterized MCI patients by Flutemetamol-PET, which is a marker for amyloidopathy and we compared DBI serum levels between prodromal AD patients (PET positive) and cognitive deteriorated subjects not presenting amyloid deposits (PET negative).

Our findings revealed higher DBI serum levels in the first group and this increase might represent an early alteration in AD pathology, which is detectable in its prodromal stage. However, the overlap in DBI serum values between amyloid-PET positive and negative cohorts indicates that this marker is not specific for AD but rather correlates with degenerative dementias.

In NDCI subjects, we measured DBI CSF levels together with AD core markers and this examination allowed us to distinguish three different subgroups, depending on their Aβ profile: AD, suspect AD and non-AD. Moreover, we also analysed DBI serum levels, which were very different between AD and healthy controls as well as between AD and non-AD. Despite the limitations deriving from the small sample size, DBI CSF levels were not very dissimilar among AD, suspect AD and non-AD. However, considering all patients together, DBI serum concentration was 52% higher in serum than in CSF.

In light of these observations, we verified the putative existence of a serum-CSF relationship and we found a direct correlation in DBI levels between the compartments. This evidence might contribute to a better understanding of central processes, given that it is easier to explore periphery rather than CNS. The DBI passage most likely occurs at the blood-brain barrier fenestrated capillaries, driven by a serum-to-CSF gradient.

In the same cohort of patients we investigated the presence of potential correlations between CSF levels of DBI and Aβ, t-tau or p-tau. We observed a positive correlation between DBI CSF levels and t-tau or p-tau, which might indicate a role for DBI as a marker of neurodegeneration without any specificity, as stated by previous literature.

Moreover, given DBI function as a $GABA_A$ inverse agonist, we examined its levels in relation to the agitation/aggression symptom in a subgroup of AD patients, selected among NDCI population. DBI levels were overlapping in AA+ and AA- patients and we did not find any correlation of DBI serum or CSF concentration with NPI-12, CBI or AA score. However, considering the small sample size, these data need to be confirmed, especially as regards CSF measurements.

In addition, we analysed DBI serum levels in a small cohort of delirium subjects that were hospitalized due to different causes and we observed that DBI was increased in delirium patients compared to both AD ones and healthy controls. Even though this result should be considered as preliminary, because of the limited number of individuals screened, it might strengthen DBI role as a $GABA_A$ inverse agonist. Indeed, more than 80% of our delirium patients showed a hyperkinetic

phenotype, which might be reasonably explained by a reduction in the GABAergic transmission. Moreover, the higher DBI serum levels observed in this cohort in comparison with the AD one might be a consequence of the acute inflammatory condition typical of delirium subjects, which discriminate them from AD patients, rather displaying chronic inflammation, and that could enhance the activation of the TSPO/DBI system. Pro-inflammatory cytokines serum measurement confirmed the acute inflammatory on-going process, since IL-6 levels were significantly higher in delirium subjects compared to AD and healthy control ones.

Thus, our data indicate that DBI increase in AD patients is not specific for this disease, since it is found in similar pathological conditions. The serum and CSF compartments seem to be somehow related but, at the moment, their association to BPSD, in particular agitation/aggression, has still to be verified.

Beyond DBI, we also analysed the TSPO receptor in lympho-monocytes (PBMC) and we quantified its gene expression by measuring the correspondent mRNA levels, which were unchanged between AD patients and healthy controls.

On the contrary, the increased expression of TSPO gene in cerebral areas affected by inflammatory and neurodegenerative processes has already been described in literature[1058]. Indeed, this receptor is used a marker for activated microglia in PET studies, in which it is employed as a target for tracers such as PK11195. Moreover, TSPO is expressed on peripheral monocytes and it has been shown to regulate their chemotaxis through the blood-brain barrier, in order to boost their migration towards inflamed brain areas where they can differentiate in blood-derived macrophages, which are able to support resident microglia impaired activity[1001].

However, our study demonstrated that the increased TSPO levels detected in the brains of our AD patients by a PK11195-PET are not associated to the induction of TSPO gene expression in PBMC. This could depend on the fact that TSPO synthesis might be activated in peripheral monocytes following their migration and exposure to a pro-inflammatory milieu. On the other hand, TSPO increased levels in the CNS might be correlated to a reduced degradation of this receptor rather than to its increased synthesis and future analyses of the total as well as membrane TSPO protein will be useful to verify this second hypothesis. Indeed, they will unravel any putative increase in TSPO receptor protein, regardless of its unchanged mRNA levels. Moreover, it has to be noticed that mRNA quantification was performed in the whole PBMC population, in which lymphocytes

are 4-fold more represented than monocytes, in order to analyse the cells immediately after their isolation from the patients and to avoid incubation times that could eliminate potential variations induced by soluble factors released in the blood. Thus, lymphocytes richness in the samples that we analysed might have masked some differences belonging to the monocytes population only.

On the other hand, we were also interested in evaluating the impact of rs6971 TSPO polymorphism on the TSPO/DBI system. Indeed, this SNP regulates PK11195 binding to the TSPO receptor and it has been associated to anxiety disturbances[1061]. Its frequencies in our AD population were congruous with the ones described in literature but we did not observe any significant difference in PBMC TSPO gene expression among the allelic sub-groups, always taking into account that its mRNA levels did not reflect directly the protein amount in our samples.

On the contrary, we identified a slight increase in DBI serum levels in the subjects expressing the A/A polymorphism compared to the ones expressing the G/G variant. However, this evidence has to be interpreted considering that the patients expressing the A/A SNP in our cohort were less represented than the others and this disparity might have influenced the absence of statistical significance. Moreover, the subjects expressing the G/A polymorphism displayed mildly increased DBI serum levels compared to the ones expressing the G/G allelic variant and this finding might be linked to the presence of the A allele. For this reasons, it might be useful to extend the study to a wider population, in order to verify if the levels of DBI in the A/A group increase enlarging the sample size.

Nonetheless, the small differences observed in DBI serum levels among the rs6971 SNP subgroups were not paralleled by significant variations in NPI-12 scores, confirming that this polymorphism does not affect BPSD manifestations.

Despite the absence of an evident induction of TSPO gene expression in PBMC of AD patients, we hypothesized that the increased serum levels of its ligand DBI could be sufficient to determine an over-stimulation of the molecular mechanisms down-stream TSPO activation, which are represented by neurosteroids and BDNF production as well as by monocytes chemotaxis.

Thus, in the attempt to study the neurosteroidogenic down-stream pathway of the TSPO/DBI system, we measured the serum levels of DHEA-S, a neurosteroid produced following cholesterol mitochondrial import driven by TSPO[1058]. Comparing AD patients to healthy controls, we did not find any difference neither in DHEA-S serum levels nor in its molar ratio with cortisol (CDR), which

is also known as *stress balance*. In a previous study, Pluchino et al. hypothesized that DHEA-S could influence cognitive performances in AD subjects, given its negative allosteric modulatory properties on the $GABA_A$ receptor, the inhibition of which has been associated to cognitive impairment[1062]. However, these results are controversial. Indeed, Leblhuber et al. found lower DHEA-S serum levels in AD patients than in healthy controls[1063], whereas Carlson et al. did not observe any significant difference between the two groups as regards DHEA-S serum levels and CDR[1064]. For these reasons, further investigations are needed to clarify the potential pathogenic role of neurosteroids in AD pathology.

Moreover, considering BDNF important role in maintaining structural integrity and function of neuronal circuits, we quantified the serum levels of this neurotrophin in both AD and control populations and we did not detect any significant difference between the two cohorts. In a previous study, Christensen et al. found reduced BDNF levels in the hippocampus and serum of AD subjects[1065] and, similarly, Laske et al. reported lower concentrations of this neurotrophin in both CSF and blood of AD patients[1066]. Nevertheless, another study did not identify any difference in BDNF serum and CSF levels between AD patients and controls[1067], questioning its precise role in the pathology.

In this study, we also analysed the rs6265 BDNF polymorphism, whose distribution in our AD population reflected the one found in literature. Even though this SNP has been associated to lower BDNF brain levels[1068] as well as to reduced hippocampal volume, increased risk for AD and worse cognitive performances, the evidences existing in literature are not conclusive. However, we did not identify any significant difference in BDNF serum levels in our AD population following its stratification for the rs6265 polymorphism and this evidence is in line with the data from Trajkovska et al.[1069].

Even though our analyses evidenced a clear difference in DBI serum levels between AD patients and healthy controls, the evaluation of the same parameter in AA+ and AA- AD subgroups showed modest results.

The initial hypothesis stated that an over-stimulation of the TSPO/DBI system, together with the activation of monocytes migration, could contribute to the development of behavioural disturbances, in light of the limited evidences showing that the activation of peripheral innate immunity in animal models could generate symptoms similar to BPSD[1070]. In this context, DBI

could sustain this process through TSPO binding, given its positive role in regulating monocytes chemotaxis[1001].
On the other hand, this activity is also exerted by benzodiazepines, which are known for inducing BPSD in demented patients.

However, the results obtained in this study indicate that neither DBI nor the substances produced by the TSPO/DBI pathway (DHEA-S and BDNF) show significant differences in their serum concentration between AA+ and AA- subjects. Moreover, DBI could represent a promising biomarker for BPSD not only because of its TSPO activating function but also due to its role as an inverse agonist of the $GABA_A$ receptor. Nevertheless, despite its higher levels in AD patients compared to healthy controls, this increase doesn't correlate with BPSD onset, at least as regards the agitation/aggression symptom.

On the contrary, we detected a small reduction in TSPO gene expression in PMBC of AA+ patients compared to AA- ones and this evidence excludes that an increased TSPO peripheral synthesis could be implicated in the genesis of agitation/aggression. In this case, the quantification of TSPO protein, including the transmembrane one, together with the putative confirmation of its reduction in agitated patients, might support the hypothesis of a correlation between reduced TSPO expression and BPSD genesis.

Pursuing the aim to identify a biomarker for behavioural disturbances, we examined irisin as a potential indicator of BPSD linked to motor hyperactivity, such as agitation/aggression. This parameter would overcome the limits connected to the employment of caregiver-based clinical scales, which are exposed to biases deriving from the personality as well as the personal expectations of the caregiver itself. The availability of unbiased BPSD biomarkers would guarantee a better identification of patients that need pharmacological therapy and would offer suitable outcomes to define the efficacy of new treatments in clinical trials. An appropriate example in this sense is represented by actigraphy, which consists in the registration of a patient's motor activity through the application of a movement sensor. These studies demonstrated that the registered motor activity correlates with the severity of the symptom reported by the caregiver[1071] and also detects positive responses to pharmacological treatments for agitation, which could not be appreciated using caregiver-based clinical scales[1072]. To date, there are no studies in literature that explore biochemical BPSD biomarkers in large AD patients cohorts.

Irisin is a myokine that is released by muscles during physical exercise. It is particularly interesting because of its stimulating action on motor activity[1073], which suggests a possible role for this molecule as a factor stimulating agitation.

As expected, our data evidenced a slight increase in irisin serum concentration in patients displaying agitation/aggression compared to AA- ones as well as to healthy controls. Moreover, a correlation between irisin serum levels and the duration of AA manifestations has been demonstrated. There are no other studies that explored irisin levels in patients affected by neurodegenerative diseases.

Our data indicate that BPSD displaying an increased motor activity lead to the release of myokines in the circulation and that the extent of this release correlates with the fraction of disease duration which follows AA onset. Thus, it seems that irisin increase is linked to the chronic expression of agitation/aggression symptom rather than to the severity of single episodes.

It has to be mentioned that the mildly increased irisin levels detected in AA+ patients, together with the wide overlap of its measurements between these subjects and AA- ones, prevent this molecule from being considered a useful biomarker to identify the presence of agitation/aggression in AD patients.

Similarly, the absence of an evident correlation between irisin serum levels and the severity of AA, expressed by NPI and CBI scores, thwarted also this parameter from being applied to define severity and nursing impact of the agitation symptoms. However, if a reduction in irisin levels were demonstrated in agitated patients after the introduction of treatments aimed at controlling this disturbance, this marker could become a useful outcome to define the efficacy of drugs therapies for controlling agitation in AD patients. Moreover, this evidence stimulates the research to find other biochemical or instrumental parameters that could be used as direct indicators to identify and quantify BPSD.

Despite serum quantifications of the TSPO/DBI system down-stream elements did not shown any alteration in AD patients compared to healthy controls, we also investigated the role of this system in monocytes migration. The chemotaxis experiments demonstrated that the monocytes isolated from AD subjects display a higher migration rate than the ones from healthy controls when stimulated by oligomeric Aβ, which represents one of the main hallmarks in AD pathology. Before testing the motility of AD patients' monocytes, we demonstrated the chemoattractant role

of this substance on myeloid leukaemia cells as well as on monocytes isolated from healthy volunteers. In the second case, Aβ displayed chemoattractant properties only when its concentration was as the same order of magnitude as the one detected in biological fluids (125 pM), whereas higher concentrations, which are normally used to perform toxicity assays, were less efficient in inducing chemotaxis, most likely because they activated cellular processes leading to monocytes death or dysfunction.

However, the stimulatory effect of oligomeric Aβ on chemotaxis might suggest a new mechanism by which this substance could contribute to neuronal damage. Indeed, since this peptide is present in the brain in early stages of AD, it could act as a trigger of neuroinflammation, recruiting monocytes in amyloid deposits-rich areas and favouring the neuronal impairment induced by the production of immune mediators.
These results confirmed the data from Zhang et al., which described an increased ability of monocytes isolated from AD patients to cross a model of endothelial barrier when stimulated by $A\beta^{1074}$.

Interestingly, Aβ-induced chemotaxis in healthy volunteers monocytes was totally abrogated by the combined administration of Aβ 125 pM and Ro5-4864, which is a well-known TSPO inhibitor and this evidence demonstrates that the cellular migration we observed was actually TSPO-dependent. Moreover, Ro5-4864 effect was comparable at the concentrations of 25 and 50 µM, most likely because the binding sites of this compound were completely saturated. As expected, this molecule did not exhibit any further inhibitory action on monocytes chemotaxis when administered alone, since its function occurs only *via* TSPO receptor and not through other pathways.

The comparison of monocytes chemotaxis between agitated and non-agitated patients represents an important future goal, since it will be helpful to define the possible contribution of alterations in peripheral monocytes activity in inducing BPSD.

Beyond investigating the role of monocytes in the processes underlying neuroinflammation, in the second part of this study we also concentrated our attention on peripheral neutrophils. Indeed, these cells have been shown to extravasate and infiltrate AD mouse models brains, where they localize near amyloid plaques[1041]. Moreover, their activation is characterized by the release of

Neutrophils Extracellular Traps that, representing NLRP3 inflammasome triggers, could stimulate it, leading to further neuronal damage[1075].

We evaluated the putative infiltration of neutrophils in APP/PS1 mice *per se* and crossed into *Nlrp3*$^{-/-}$ ones, in order to obtain APP/PS1/*Nlrp3*$^{-/-}$ mice, which were incapable of assembling the inflammasome. Heneka et al.[1045] have already shown the influence of this complex on cognitive performances in AD and our NLRP3-deficient model allowed us to unravel inflammasome potential contribution to peripheral cells migration in the pathology.

Our results showed that neutrophils brain infiltration was totally absent in WT as well as *Nlrp3*$^{-/-}$ animals, regardless of their age. This finding is reasonable considering that these models displayed a healthy phenotype, without any evidence of previous cerebral damage, in both their young and old age. Moreover, even though we injected some of them with LPS to induce systemic inflammation, we did not observe any cellular migration in these LPS-treated animals as well. We might speculate that one LPS injection is not sufficient to stimulate neutrophils infiltration in the brain, since it represents a single trigger in a healthy background. This theory has already been proposed as regards microglia, which need priming before activating exaggerated inflammatory responses following a systemic damaging stimulus[1076]. Similarly, neutrophils might exert their functions in periphery following LPS injection but, being in a healthy environment, they might not be primed and prone to activate excessive immune reactions leading to their extravasation. Also, the absence of the inflammasome in *Nlrp3*$^{-/-}$ animals might favour this limited immune response, given that this complex normally activates the production of IL-1β, which is one of the most effective cytokines in boosting strong inflammatory responses.

On the contrary, we observed neutrophils infiltration in the brains of both APP/PS1 as well as APP/PS1/*Nlrp3*$^{-/-}$ mice and this phenomenon was detected in close proximity to amyloid plaques only. Indeed, we did not identify any positive cell in plaque-free areas, confirming the previous findings from Zenaro et al.[1041], which showed neutrophils presence in mouse brain parenchyma in Aβ deposits-rich regions. Moreover, considering each genotype *per se*, we found higher numbers of infiltrated neutrophils in old animals than in the young ones and this is most likely due to the increased amyloid deposition that occurs in the brain as the underlying AD pathology progresses. In support of this assumption, Zenaro et al.[1041] demonstrated that Aβ is responsible for the adhesion of neutrophils to vessels endothelium and this process is considered essential for their migration.

On the other hand, regardless of their genotype, LPS-treated animals displayed a huge neutrophils infiltration compared to the CTRL ones. This might also depend on our previous priming hypothesis and, in this case, a single injection of LPS superimposed on an AD pathological condition might represent a second trigger that could activate several mechanisms driving neutrophils migration. It is interesting to note that, in both APP/PS1 as well as APP/PS1/*Nlrp3*$^{-/-}$ mice, the infiltration of peripheral neutrophils was higher in animals sacrificed 10 days after LPS administration rather than in the ones sacrificed 2 days post-LPS injection. This finding is somehow controversial, since 10 days after the LPS stimulus the recovery mechanisms should have already started to heal the tissue. However, it has to be considered that the genetic background of our animals is pathological and homeostatic mechanisms might be easily dysfunctional. Thus, the strong neutrophils migration that occurs after 2 days might induce a chronic inflammatory response sustained by the production of specific mediators which, in turn, attract to the CNS other peripheral cells. This speculation is further strengthened by the increased KC-GRO levels that Heneka's research group found in the brains of the same LPS-treated animals (unpublished data). Indeed, this molecule is considered to be the main chemokine driving neutrophils chemotaxis.

As regards the comparison between APP/PS1 and APP/PS1/*Nlrp3*$^{-/-}$ mice, we detected an increase in neutrophils infiltration in old LPS-treated NLRP3-deficient animals but this evidence deserves more detailed studies. Indeed, it seems in contrast with the findings from Heneka et al. which demonstrated that *Nlrp3*$^{-/-}$ animals display decreased amyloid amounts and deposition[1045]. Nevertheless, the available data are not enough to hypothesize a congruent explanation for this discrepancy and other analyses will be necessary. On the other hand, it has to be considered that we identified only activated neutrophils through their NETs expression but we might have missed a non-activated sub-population that could be detected using other markers, such as Gr-1 and that could be useful to clarify the whole scenario regarding neutrophils infiltration.

Chapter 10

Conclusion

10.1 Conclusion of the Study

In this study we analysed several components of the TSPO/DBI system and we investigated their potential alterations in AD pathology, in order to unravel any possible influence of these variations in BPSD genesis, through the regulation of neurosteroidogenesis and monocytes chemotaxis. Moreover, we examined the contribution of peripheral neutrophils to AD brain inflammation.

Our findings demonstrated that, despite the unchanged TSPO gene expression in the disease, the endogenous ligand of this receptor, DBI, shows clear variations in AD patients, suggesting its possible role as biomarker. However, we verified that this molecule is also altered in MCI as well as in other disorders displaying cognitive deterioration and in delirium, underlining its aspecificity for AD pathology and raising the possibility to use it as a marker for degeneration in wider terms. Also, this assumption is further strengthened by CSF DBI correlation to AD-core markers of neurodegeneration t-tau and p-tau, rather than Aβ.

On the other hand, we revealed a potential communication between serum and CSF, highlighted by the correlation of DBI levels between the two compartments. However, this association seems not to be linked to the agitation/aggression symptom that, up to date, lacks unbiased biomarkers useful for its identification. In the attempt to determine one, we examined the myokine irisin, which has been associated to physical activity but the results in this sense were not definitive.

As concerns the influence of peripheral inflammation in AD, we examined both peripheral monocytes as well as neutrophils, confirming their altered TSPO-dependent chemotaxis in the first case and their infiltration in AD mice brains in the second one.

However, investigating different pathologies by means of various tests generated discrepancies in our data, thus future experiments will be aimed to solve the biases existing in this study. Also, they will explore more widely the TSPO/DBI system in its multiple functions, in order to elucidate its potential contribution to BPSD genesis.

In particular, imaging studies will be introduced, in order to highlight any potential involvement of alterations in different cerebral areas in the development of neuropsychiatric symptoms. Also, we will correlate DBI serum levels in AD patients with the amplitude of neuroinflammation determined by means of PK11195 PET, with the aim to verify if this peptide could represent a peripheral biomarker of this central process.

On the other hand, we will investigate DBI concentration in different non-AD dementia conditions and we will examine the association between DBI potential increase and cortical atrophy distribution profiles or cortical hypometabolism, determined by MRI and FDG-PET, respectively, in both AD and non-AD demented subjects, in order to define any putative topographic specificity of DBI as a neurodegeneration marker.

In addition, we will quantify TSPO protein on PBMC from AA+ and AA- AD subjects as well as healthy controls, to highlight any possible difference that did not emerge from the mRNA levels quantification and to verify if the decreased TSPO gene expression observed in AA+ group is accompanied by TSPO protein reduction as well.

Moreover, we will analyse monocytes chemotaxis in the AD population dichotomized for AA symptoms not only with chemoattractants but also with TSPO antagonists, to examine if the increased motility of patients' monocytes actually derives from an over-stimulation of the TSPO/DBI system.

Considering AA symptoms, we will measure irisin concentration in AA+ patients before administering drugs against agitation, to verify the usefulness of this molecule as a surrogate biomarker to evaluate the efficacy of the pharmacological therapy in agitated AD subjects. On the other hand, we will stratify these patients for symptoms different from AA, in order to search for biomarkers related to depression and apathy.

Lastly, we will analyse the effect of cognitive reserve on BPSD onset, frequency and severity. This parameter will be quantified in relation to both education years and other questionnaires examining the intellectual daily stimulation of the patients.

Indeed, these wide spectrum analyses are necessary, since the biological basis of BPSD are still unknown and there is a huge need to clarify them, so that a proper pharmacological therapy could be administered to suffering patients. A better therapy, arising from a deeper understanding of the mechanisms driving these neuropsychiatric disturbances, could lead to their desirable reduction in diseased cohorts, which, in turn, results in the improvement of patients' and caregivers' quality of life as well as in a decreased economic burden for the healthcare system.

Chapter 11

Appendix

11.1 Beyond AD

Even though BPSD are very frequent and common in Alzheimer's Disease, this is not the only pathology displaying these symptoms. Indeed, other forms of dementia have been associated to behavioural disturbances and, among them, the best-characterized is Fronto-Temporal Dementia (FTD), which consists in frontal as well as temporal brain lobes neurodegeneration and atrophy, leading to personality and mood changes, language alterations or aphasia.

Interestingly, in recent years FTD has been shown to share some clinical features with Amyotrophic Lateral Sclerosis (ALS) that, in turn, is often accompanied by fronto-temporal cognitive deficits[1077]. This concept has taken a while to be accepted, since ALS has always been considered a progressive neurodegenerative disorder involving motor-neurons but not cognitive functions. On the contrary, it is now clear that these processes are impaired in ALS and that this pathology presents neuropsychological alterations as well[1078–1082]. For these reasons, a *continuum* between FTD and ALS has been hypothesized and some intermediate pathological forms have been recognized[1083,1084]. Among them, ALS-ci (cognitive impairment) and ALS-bi (behavioural impairment) are particularly interesting, because they strengthen the link between a motor-neurons disease and several clinical features typical of dementia, raising the possibility to identify a common aetiological pathway in neurodegeneration. The ALS-ci variant is characterized by cognitive dysfunctions in the frontal lobe but it doesn't normally display mnemonic deficits or motor patterns alterations[1085], whereas ALS-bi exhibits behavioural disturbances such as apathy or depression but not cognitive impairment[1086–1088]. Both forms deserve clinical attention, especially in light of important choices regarding life-ending therapies for ALS subjects. Indeed, if they are affected by not only motor impairments but also dementia-like symptoms, they might not be able to evaluate properly their situation and to make the best decisions for themselves and their families. Moreover, the additional presence of behavioural problems in ALS patients often intensifies the stress burden of the caregivers, increasing the risk for their burn-out[1089,1090].

Considering the recently discovered role of neuropsychological symptoms in motor-neurons disease, we decided to extend our study on the TSPO-DBI system to an ALS population, with the aim to highlight any potential alteration of this pathway in these subjects. In order to do that, we recruited n=54 ALS patients as well as n=25 sex- and age-matched healthy controls (CTRL) (for the details about the populations see **Table 8**), which were screened for DBI serum levels and TSPO

gene expression. The diseased cohort was evaluated by means of clinical tests like ALSFRS-r (Amyotrophic Lateral Sclerosis Functional Rating Scale-revised), DPI (Disease Progression Index), FAB (Frontal Assessment Battery) and MoCA (Montreal Cognitive Assessment). Moreover, a subgroup of patients was assessed for cognitive (n=30) and behavioural (n=26) impairment using ALSCBS-ci and ALSCBS-bi (ALS-Cognitive Behavioural Screen), respectively.

	CTRL (n=25)	ALS (n=54)
Sex (M/F)	17/8	40/14
Age (ys)	62.7 ± 12.5 (39-86)	63 ± 11.5 (38-88)
Duration (mo)	N/A	45.9 ± 43 (7-236.5)
Onset location (S/B)	N/A	49/5
ALSFRS-r	N/A	30 ± 9.2 (10-47)
DPI	N/A	0.58 ± 0.36 (0.3-1.5)
FAB	N/A	14.7 ± 3 (3.8-18)
MoCA	N/A	21.5 ± 4.4 (8.7-30)
ALSCBS-ci	N/A	13.8 ± 4.4 (4-19)
ALSCBS-bi	N/A	32.1 ± 9.5 (12-45)
PEG (Y/N)	N/A	7/47
NIV (Y/N)	N/A	21/33
Riluzole, 50 mg bid (Y/N)	N/A	8/46

Table 8: Complete list of the clinical, demographic and social information about ALS and CTRL recruited populations. ALSCBS-ci and ALSCBS-bi were collected in a subgroup of 30 and 26 patients, respectively (mean ± SD, range) (N/A: not applicable; range intervals in brackets).

As regards the results we obtained, we observed a higher DBI serum concentration in ALS patients than in healthy controls (5-fold increase, $p<0.001$) (**Fig. 42**). On the other hand, even though DBI serum levels did not show any correlation with ALSFRS-r or disease duration, they displayed a negative correlation with DPI ($r=-0.29$, $p= 0.03$) (**Fig. 43**). However, this association was lost correcting the analysis for multiple correlations. Moreover, dividing ALS patients for disease progression velocity, according to the cut-off in Kimura et al.[1091], we did not find any significant difference in DBI serum concentration among the subgroups (data not shown).

Also, we sorted our ALS cohort by FAB as well as MoCA scores but we did not identify any difference in DBI serum levels between the group below and the one above the cut-off value of these tests (data not shown).

Similar results were obtained separating ALS patients depending on their ALSCBS-ci score. This test was administered to 30 subjects only and 23.3% of them displayed pathological scores (ALSCBS-ci+) but DBI serum concentration in this group did not show any significant change compared to the one in the non-pathological individuals (ALSCBS-ci-) (**Fig. 44**).

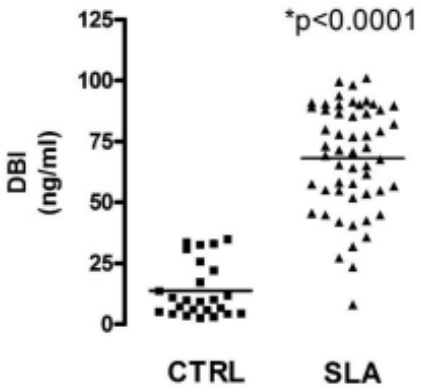

Figure 42: DBI serum levels in ALS patients and healthy controls. DBI concentration displays a 5-fold increase in ALS patients compared to CTRL (***p<0.0001, two-tailed Student's t-test, mean ± SD).

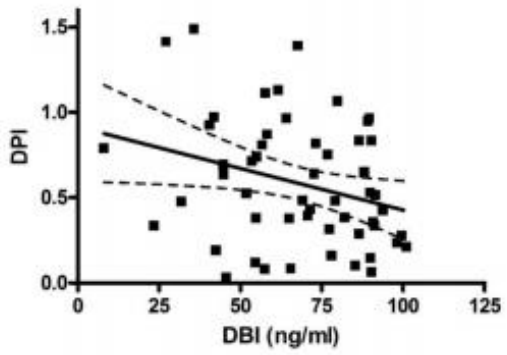

Figure 43: Inverse correlation between DBI serum levels and DPI in ALS patients. Higher DBI serum levels are correlated to a slower-progressing disease (*p=0.03). This correlation is lost correcting the analysis for multiple correlations.

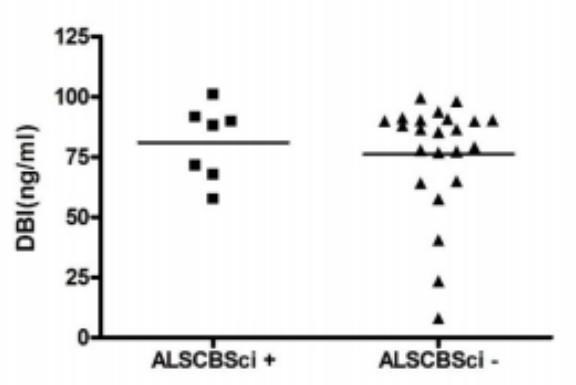

Figure 44: DBI serum levels in a subgroup of ALS patients dichotomized according to the ALSCBS-ci score (n=30). No differences are found in DBI serum concentration between the pathological group (ALSCBS-ci+) and the non-pathological one (ALSCBS-ci-) (two-tailed Student's t-test, mean ± SD).

On the contrary, we observed an inverse correlation between DBI serum levels and ALSCBS-bi scores (r=-0.44, p<0.03), which were registered in a subgroup of 26 patients (**Fig. 45**).

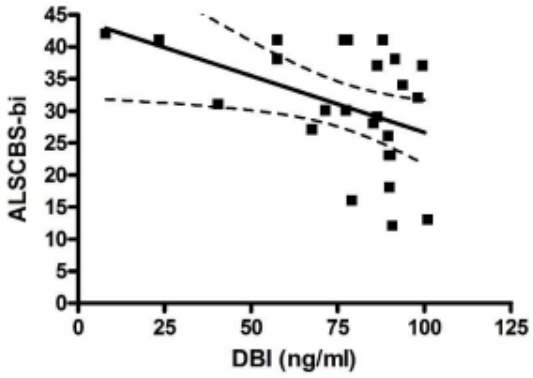

Figure 45: Inverse correlation between DBI serum levels and ALSCBS-bi scores in a subgroup of ALS patients (n=26). Higher DBI serum levels are correlated to lower ALSCBS-bi scores (*p<0.03, n=30).

Moreover, dichotomizing these subjects for the presence (ALSbi) or absence (ALSnobi) of behavioural anomalies, according to the cut-off in Woolley and Katz[1092], we detected a higher serum concentration in the first cohort than in the second one (**Fig. 46**).

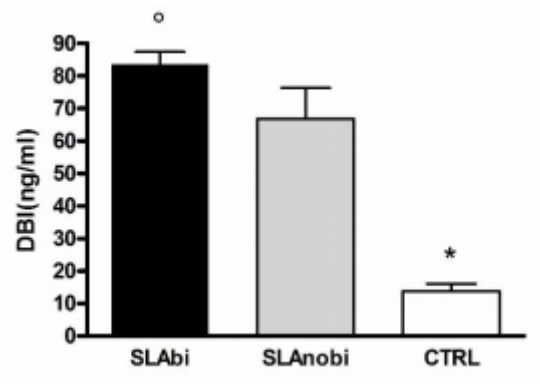

Figure 46: DBI serum concentration in a subgroup of ALS patients dichotomized for the presence or absence of behavioural anomalies (n=26). The subgroup displaying behavioural anomalies (ALSbi) shows a higher DBI serum concentration compared to the one without behavioural disturbances. DBI serum levels in both ALS subgroups are significantly higher than the ones in the control group (***p<0.0001, one-way ANOVA, followed by Newman-Keuls post-hoc test; *p<0.001 vs. both ALS groups; °p<0.05 vs. ALSnobi).

Lastly, we measured TSPO gene expression in PBMC of 20 ALS patients as well as 16 CTRL and we observed an increase in the mRNA of this protein in the diseased subjects compared to the healthy ones (3-fold increase, p<0.0001) (**Fig. 47**). However, this data did not show any correlation to DBI serum levels in the same populations. No changes were found between ALS and CTRL subjects in TSPO activation by-products DHEA-S and cortisol (**Fig. 48A, B**).

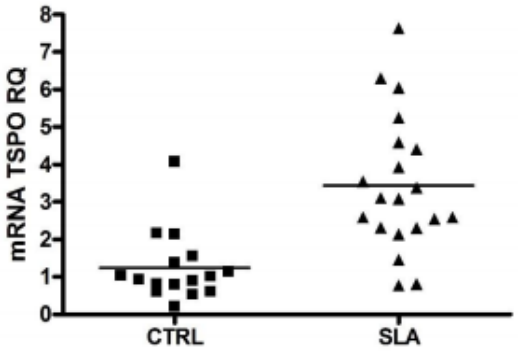

Figure 47: TSPO gene expression in PBMC of ALS patients and healthy controls subgroups. TSPO gene expression is increased in ALS patients compared to healthy controls (3-fold increase, ***p<0.0001). Relative Quantification (RQ) of TSPO mRNA is calculated as ratio to β-actin (two-tailed Student's t-test, mean ± SD).

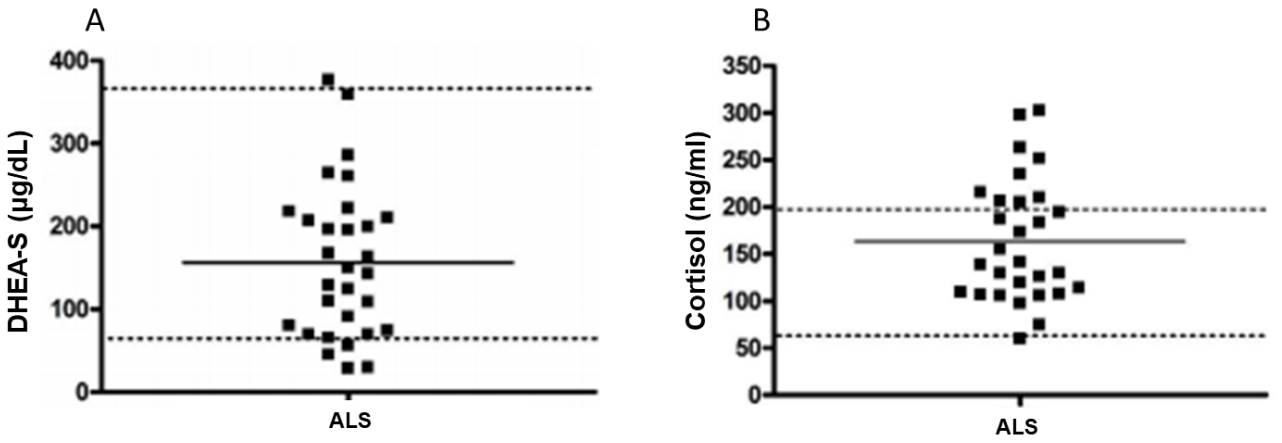

Figure 48: DHEA-S and cortisol serum levels in ALS patients. (A) DHEA-S serum levels in ALS patients are unchanged compared to healthy controls (not represented, average range from analyses laboratory: 65-368 µg/dL). **(B)** Cortisol serum levels in ALS patients are unchanged compared to healthy controls (not represented, normality range from analyses laboratory: 62-194 ng/ml). (two tailed Student's t- test, mean ± SD).

Our analyses on the TSPO/DBI system were aimed to identify a potential unbiased biomarker that could be used to detect behavioural anomalies in ALS patients, independently from the employment of caregiver-based clinical scales. Indeed, even though the validity of these instruments has already been demonstrated[1093], they could lead to an under- as well as over-estimate of behavioural disturbances in ALS subjects.

Our results indicate that DBI serum levels are increased in the ALS cohort compared to the healthy controls but the findings previously reported in this study showed that the same increase was detected also in our AD population, suggesting that DBI might represent an aspecific marker of degeneration.

Moreover, DBI serum levels do not display any correlation with ALSFRS-r scores or disease duration but they exhibit an inverse correlation with DPI. However, this association is lost following patients' stratification according to Kimura and colleagues[1091]. Similarly, we did not observe any significant difference in DBI serum concentration dichotomizing ALS patients according to their ALSCBS-ci scores.

On the contrary, we identified a negative correlation between DBI serum levels and ALSCBS-bi scores, which indicates that the serum concentration of this peptide increases in parallel with behavioural manifestations. This association is maintained stratifying our cohort in a subgroup

displaying behavioural anomalies and another one not displaying them, according to Woolley cut-off[1082].

Concerning the TSPO/DBI system, we also measured TSPO gene expression in PBMC and we observed increased TSPO mRNA levels in ALS patients compared to healthy controls. Even though this evidence might suggest an up-regulation of TSPO receptor, we are not able to confirm this hypothesis, given that the data reporting TSPO protein expression are not available to date.

In light of these findings, it is fascinating to hypothesize that DBI could represent a marker for behavioural disturbances and, perhaps, might contribute to their genesis. Moreover, the entire TSPO/DBI system could be dysfunctional. However, our data in ALS are preliminary and prevent us from excessive speculations. Further studies are needed, in order to clarify the role of this system in a severe condition which is no longer considered only a motor-neurons disease but that is now recognized in its various forms, including the ones displaying cognitive and behavioural anomalies.

Chapter 12

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