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PHENOTYPIC HETEROGENEITY IN ALZHEIMER'S DISEASE: THE STUDY OF MOLECULAR FACTORS INVOLVED IN GENERATION OF DIFFERENT CLINICAL-PATHOLOGICAL PHENOTYPES

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Phenotypic heterogeneity in Alzheimer's Disease: the study of molecular factors involved in generation of different clinical-pathological phenotypes

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Università degli Studi di Milano-Bicocca

School of medicine and surgery

Ph.D. Thesis

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Abstract

Alzheimer's disease (AD) is the most common form of dementia among elderly. It is characterized by progressive loss of memory and other deficits in cognition and behaviour that inevitably affect self-care. Until now, AD was often conceptualized as a unitary clinico-pathological entity. However, in the last few years, several studies led to the recognition of distinct clinical and neuropathological phenotypes, spreading the view of AD as a highly heterogeneous disorder. Recent studies revealed that Aβ assemblies might have structural differences among AD brains and that such pleomorphic assemblies can correlate with distinct disease phenotypes. Neuroinflammation is a fascinating and not yet fully explored aspect of AD. The role of neuroinflammation in AD pathogenesis was highlighted after the discovery that mutations in genes encoding immune receptors increase the risk to develop AD. However, the involvement of neuroinflammation in the generation of different clinicopathological profiles of AD in unknown. Gene profile studies may offer grounds to explore such involvement. One of the objectives of this study was therefore to test whether some players of neuroinflammation, with particular attention to microglia and some cytokines, are relevant for the development of distinct AD phenotypes. Aim of this project was to study the main molecular mechanisms involved in the generation of different clinical/neuropathological phenotypes of the disease and possibly, provide grounds for a molecular classification of the disease. Following an approach similar to that largely used in the study of prion diseases, a multidisciplinary strategy has been chosen in order to:

1. Identify molecular profiles based on the content of Aβ peptides in brains from AD patients with different clinical and neuropathological phenotypes.

2. Test the hypothesis that some players of neuroinflammation (microglial cells and immunocytokines) are involved in the determination of clinical and pathological phenotypes of AD.

We found that in both sporadic and inherited forms of AD, amyloid aggregates differ in the biochemical composition of Aβ species. These differences affect the physicochemical properties of Aβ assemblies including aggregation kinetics, resistance to degradation by proteases, seeding ability and capability to induce distinct patterns

of Aβ-amyloidosis when injected into mice. Concerning microglia, we found that are differently represented within the brain of patients affected by distinct clinic-pathological forms of AD and that such differences concern morphology, distribution and density. We observed that overall levels of the neuroinflammatory molecules analyzed are significantly higher in AD samples than controls, regardless of their pro or anti-inflammatory effect. Moreover, results revealed that it is possible to isolate different neuroinflammatory subgroups through the relative quantity of different neuroinflammatory molecules. Among the molecules analyzed, CXCL13 resulted significantly higher in AD patients compare to non-demented subjects. ELISA assay confirmed the relevance of this result also in cerebrospinal fluid (CSF) while in plasma only a similar trend was observed. Overall these data suggest that the distinct mixtures of Aβ seeds with distinctive physicochemical and biological properties lead to the generation of distinct AD molecular subgroups. In the same way, is it possible to identify neuroinflammatory profiles in AD patients, based on inflammatory molecules secreted by microglia. These profiles seem to be not in accordance with those identified by Aβ. Finally, the results of these study nominate CXCL13 as a potential biomarker of neuroinflammation in AD, but further investigations are required to fully characterize its implications in the pathology and its usefulness in the diagnostic protocols for the disease.

Abstract (Italian)

La malattia di Alzheimer (MA) è la forma più comune di demenza tra gli anziani. È caratterizzata da una progressiva perdita di memoria, deficit cognitivi ed alterazioni comportamentali. Fino ad ora, la MA è stata pensata come un'entità clinico-patologica unitaria. Tuttavia, negli ultimi anni, diversi studi hanno portato al riconoscimento di fenotipi clinici e neuropatologici distinti, suggerendo una nuova visone della malattia come altamente eterogenea. Studi recenti hanno rivelato che gli aggregati di Aβ presenti nei cervelli di diversi pazienti Alzheimer potrebbero presentare caratteristiche strutturali differenti e che tali gruppi pleomorfi potrebbero essere correlati con fenotipi distinti della malattia. La neuroinfiammazione è un aspetto affascinante e ancora poco esplorato della MA. Il ruolo della neuroinfiammazione nella patogenesi della malattia è supportato dalla scoperta di mutazioni geniche, codificanti per i recettori immunitari, associate ad un aumentano rischio di sviluppare la MA. Tuttavia, il coinvolgimento della neuroinfiammazione nella generazione di diversi profili clinico-patologici della MA è tuttora sconosciuto. Lo Scopo di questo progetto è stato quello di studiare i principali meccanismi molecolari coinvolti nella generazione di diversi fenotipi clinici/neuropatologici della patologia e possibilmente fornire i presupposti per una classificazione molecolare della malattia. Seguendo un approccio simile a quello largamente utilizzato nello studio delle malattie da prioni, è stata scelta una strategia multidisciplinare al fine di:

1. Identificare i profili molecolari basati sul contenuto dei peptidi Aβ nel cervello di pazienti con differenti fenotipi clinici e neuropatologici di MA.

2. Verificare l'ipotesi che alcuni attori della neuroinfiammazione (cellule microgliali e citochine) siano coinvolti nella determinazione dei fenotipi clinici e patologici della MA.

Abbiamo trovato che in entrambe le forme sporadiche ed ereditarie della MA, gli aggregati di amiloide differiscono nella composizione biochimica delle specie di Aβ. Queste differenze influenzano le proprietà fisico-chimiche degli aggregati di Aβ tra cui la cinetica di aggregazione, la resistenza alla degradazione da parte delle proteasi, la capacità di "seeding" e quella di indurre profili distinti di Aβ-amiloidosi quando iniettati nei topi. Riguardo la microglia, abbiamo scoperto che essa è differentemente rappresentata all'interno del cervello di diversi pazienti Alzheimer e che tali differenze riguardano morfologia, distribuzione e densità. Abbiamo osservato che i livelli complessivi delle molecole neuroinfiammatorie analizzate sono significativamente più alti nei campioni di MA rispetto ai controlli, indipendentemente dal loro effetto pro o anti-infiammatorio. Inoltre, i risultati hanno rivelato che è possibile

isolare diversi sottogruppi neuroinfiammatori attraverso la quantità relativa delle diverse molecole neuroinfiammatorie. Tra le molecole analizzate, la chemochina CXCL13 è risultata significativamente più alta nei pazienti con MA rispetto ai soggetti non dementi. Il saggio ELISA ha confermato la rilevanza di questo risultato anche nel liquido cerebrospinale (CSF) mentre nel plasma è stata osservata solo una simile tendenza. Nel complesso questi dati suggeriscono che le distinte miscele di peptidi Aβ con proprietà chimico-fisiche distintive generano sottogruppi molecolari di MA distinti. Allo stesso modo, è possibile identificare profili neuroinfiammatori, basati su molecole infiammatorie secrete dalla microglia. Questi profili sembrano non essere conformi a quelli identificati dai peptidi Aβ. Infine, i risultati di questi studi candidano CXCL13 a potenziale biomarker neuroinfiammatorio nella MA. Tuttavia, sono necessarie ulteriori indagini per caratterizzare pienamente le sue implicazioni nella patologia e la sua utilità nei protocolli diagnostici per la malattia.

Contents

1 Introduction

1.1 Definition

"Alzheimer's disease (AD) is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills, and eventually the ability to carry out the simplest cognitive tasks" [7]. The name of the disease derives from its discoverer, Alois Alzheimer, a German psychiatrist and neuropathologist who firstly described its symptoms and neuropathological aspects in 1907 [6]. AD is the most common form of dementia that affects older individuals and together with Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS), is considered one of the most complicated neurodegenerative disorder. AD is characterized by specific and progressive neuronal death with reduction of the encephalic volume and intracerebral accumulation of abnormal β-amyloid (Aβ) and tau proteins which affect above all, the associative areas of the cerebral cortex, the hippocampus [165] and the trans-entorhinal cortex [236]. The selective deposition of these proteins in specific brain areas is probably responsible for the damage of distinct neuronal populations, that cause a variable spectrum of symptoms, divided in cognitive, motor or mixed alterations. Usually, symptoms at onset include episodic memory deficit. Subsequently, severe declarative and non-declarative memory impairment occur, cognitive and behavioural changes become evident [236, 229] and, in the late stage of the disease, impairment in motor functions lead the patient to the vegetative state and death. The onset of the disease is difficult to characterize, because the pathophysiological changes can precede clinical symptoms of several years or even decades [258]. The use of cognitive tests, imaging analysis and cerebrospinal fluid examination in the diagnostic protocol make possible to achieve a diagnosis with 90% of accuracy but the post-mortem brain examination remains the only way to make a definitive diagnosis of the disease. Up to now, no efficient treatments are available for this disease.

1.2 Epidemiology

The social and economic impact on the global society has made AD one of the most alarming health-related problems of the 20th century. AD represents 60%-70% of all cases of dementia accounting for about 50 million patients worldwide. The costs

for the care of these patients, mostly provided by families, is about $818 billions [273]. Dementia is the 7th leading cause of death in the world, however, real numbers could be higher than those estimated until now. Indeed some patients never receive a diagnosis and many others are accounted as dementia related deaths while the underlying cause, AD, is often not reported [124]. The most worrying data concern the future prospections: the prevalence rate of the disease grows with age, increasing exponentially after 65 and becoming 15 times greater at the age of 85 [69]. According to the longitudinal study of Wevue J. and colleagues, adult deaths among individuals with AD will increase from 32% in 2010 to 43% in 2050 [272]. These data, associated with the growth of the world's elderly population, could makes AD the 3rd leading cause of death in the world after cancer and cardiac diseases. Evaluations on the global impact of Dementia was performed by Alzheimer's Disease International (ADI) in 2015. According to this study, there were 46.8 million people worldwide living with dementia in 2015 and this number will reach 131.5 million in 2050. ADI estimated in one every 3 seconds the rate of new AD cases, and a rate of incidence of 7.7 million new cases each year [206] (Figure 1.1).

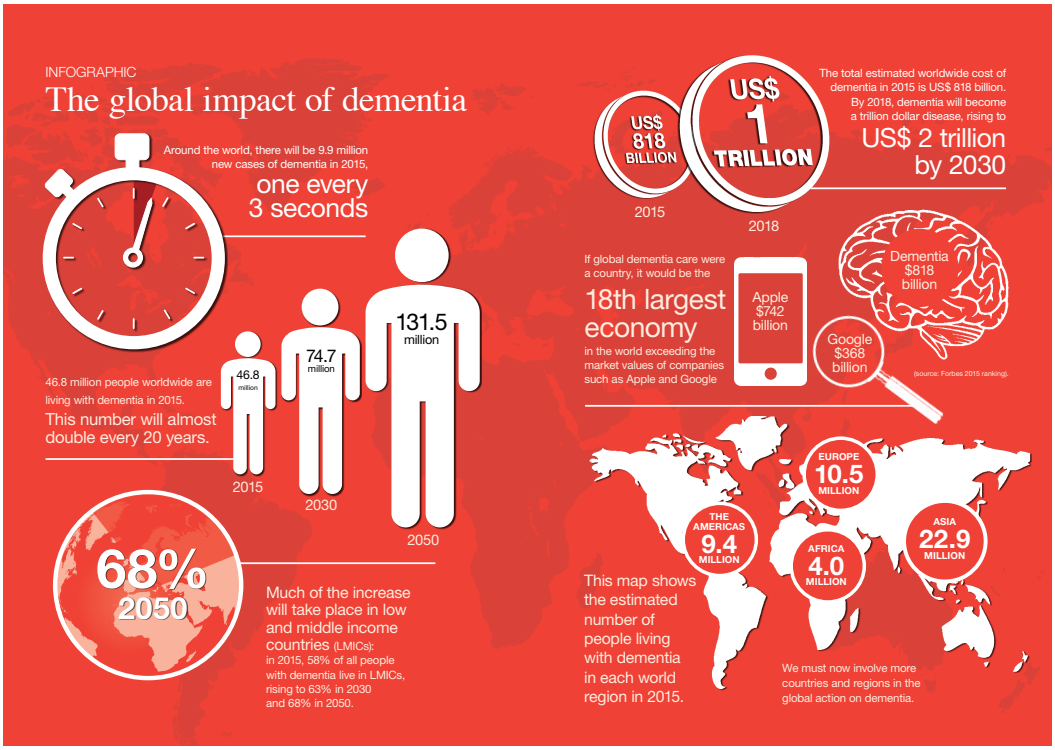

Fig. 1.1: Alzheimer Report 2015 The Global Impact of Dementia an analysis of prevalence, Incidence, cost and trends.

The prevalence of AD seems to change not only along with age but also among geographical regions. According to the "10/66 Dementia Research Group" study, the prevalence of dementia in seven different developing countries varies widely from less than 0.5% to more than 6%, substantially lower than that reported in developed countries [215]. The prevalence rate of dementia in India and in Latin America is

roughly a quarter of that of the European countries. In addition, the prevalence may be higher among the African Americans and Hispanic populations residing in the U.S.A respect to the native African populations for reasons that remain unknown [108, 181].

1.3 Pathogenesis

AD pathogenesis is not yet fully understood. Several hypotheses have been postulated during the last decades to clarify the molecular events leading to the disease. Among them, the highest credit in the scientific community was achieved by the amyloid cascade hypothesis, the tau hypothesis, the cholinergic hypothesis and the metabolic hypothesis.

1.3.1 Amyloid cascade hypothesis

Amyloid precursor protein is a ubiquitous membrane glycoprotein and is codified by the APP gene [135]. The role of this protein is still unclear, but it seems to be important in the regulation of neurite development, in neuronal survival and synaptic plasticity. A consistent fraction of APP molecules are transported to the presynaptic termination, thus of APP could be a surface receptor involved in the intracellular signal transduction, upon the binding of a ligand [163]. APP protein can undergo two proteolytic processing pathways:

1. the non-amyloidogenic pathway, in which the α-secretase firstly cleaves the transmembrane protein, generating a soluble APP fragment (sAPPα) and a membrane-bound C-terminal fragment (αCTF);

2. the amyloidogenic pathway, where the β-secretase (an aspartyl-protease, called BACE1, β site APP Cleaving Enzyme) is the first APP cutting enzyme. Thus, a different peptide called sAPPβ is generated, together with a second membrane-bound C-terminal fragment (βCTF) or C99. The γ-secretase then cuts the C99, releasing in the extracellular space the pathogenic APP fragment called Aβ peptide. The γ-secretase intra-membrane cutting site is variable and can occur after amino acids 28-40/42 originating peptides with different length. The most represented peptides are Aβ-40 (the more abundant and more soluble) and Aβ-42, more insoluble and thus more prone to aggregate.

The amyloid hypothesis, which is still considered the most probable, assumes that the neuronal degeneration and the neuropathological, functional and cognitive changes in AD are mainly caused by the generation of Aβ assemblies, namely oligomers,

which have neurotoxic properties and can promote hyper-phosphorylation of tau protein that in turns can accumulate inside neurons and is harmful for synapses. Masters and colleagues were the first to hypothesize that the accumulation of this particular protein fragment was linked with AD onset after noticing that all the brains coming from AD patients were characterized by an increased presence of Aβ [161]. This hypothesis was then corroborated by the identification of the first genetically determined form of AD, where the mutated gene was exactly the APP [80]. Furthermore, it has been noticed that individuals affected by the Down syndrome, which carry three copies of the chromosome 21, where the APP gene is located, are more prone to develop AD [105, 153]. Overall evidences led the scientific community to think that the accumulation of Aβ was the main player in the disease. However, in the last years, some studies have questioned this theory, highlighting the lack of quantitative and spatio-temporal correlations between the accumulation of β-amyloid plaques and the neuronal loss that runs parallel with the development of cognitive-behavioral impairments [210, 177, 233].

1.3.2 Tau hypothesis

Tau is a highly soluble microtubule-associated protein (MAP). In the adult human brain six isoforms of this protein have been identified, ranging from 352 to 441 amino acids, derived from the alternative splicing of a single MAPT gene located on the long arm of chromosome 17. The isoforms are distinguished by the different number of repetitions in the genic sequence which constitutes the microtubule binding domain (MT-binding) and by the variable presence of small insertions at the N-terminus [8, 179]. Tau protein has two main functions: it promotes the assembly of the microtubular network and stabilizes its structure [268]. Tau protein activity is under kinases and phosphatases regulation and several enzymes, like the mitogen-activated protein kinase (MAPK), the cyclin dependent kinase (CDK) 5 and the glycogen synthase kinase (GSK) 3P, as well as Aβ, are implicated in its phosphorylation [212, 98]. Phosphorylation of the Tau protein leads to a lower affinity between the protein and microtubules, decreasing thus their stability [4]. In pathological conditions, such as in AD pathology, the protein undergoes hyperphosphorylation, determining its detachment from microtubules and thus inducing their disorganization. At the same, hyperphosphorylated tau acts as nucleation site for protein aggregation, leading to paired helical filaments (PHF) and neurofibrillary tangles (NFTs) formation. In AD, only a portion of the hyperphosphorylated tau aggregates, losing the ability to bind tubulin to promote microtubules assembly. The remaining portion of cytosolic tau protein, does not form aggregates but loses the ability to bind tubulin altering the structure of microtubules, and actively inhibits their formation [4]. These events, which alter the cytoskeletal structure of the cell, impair neuronal functions, leading to synaptic dysfunction and neurodegeneration

[217]. The abnormal hyperphosphorylation in AD brains may be the result of an imbalanced phosphorylation/dephosphorylation system, while Tau aggregation may be due to an inefficient clearance of the misfolded protein by the ubiquitin proteasome system [42]. Up to now, hyperphosphorylation of Tau protein, together with β-amyloid accumulation, is one of the few histological signs associated to AD. However, this etiopathogenetic theory is not able to unravel the reason why there are not genetic forms of AD associated to MAPT gene mutation. Indeed, very interestingly, mutations in MAPT gene are causative of Frontotemporal Dementia (FTD) with Parkinsonism (FTDP) related to chromosome 17 [84, 202]. At the same time, amyloid plaques poorly correlate with cognitive impairment and removal of Aβ plaques by immunotherapy does not prevent neurodegeneration suggesting that Aβ cannot be the unique responsible of cognitive decline in AD [147]. In vitro studies on murine hippocampal neurons treated with Aβ showed no signs of degeneration following the deprivation of the tau protein [213]. Moreover, in knockout mice for the tau protein the presence of hyper-phosphorylated tau is necessary to mediate the synaptic and neuronal toxicity Aβ-induced [131]. Similarly, results from the AD neuroimaging studies show that cognitive decline associated with the presence of Aβ in healthy elderly individuals, occurs only when elevated levels of hyperphosphorylated tau in CSF are present [53]. Additionally, in subjects where hyperphosphorylated tau levels are low, high levels of Aβ in the CSF do not produce a significant clinical decline [53]. These data strongly suggest that Aβ requires the tau protein to mediate toxic effects. However, it is still unclear in which way tau is involved in Aβ toxicity. It is known that Aβ can increase the phosphorylation of tau protein especially in sites relevant for AD [213]. Aβ activates also caspases that can cleave the tau protein, producing truncated forms that potentially initiates or accelerates its aggregation to form NFTs [41]. Recently, Tokutake and colleagues demonstrated in vitro that an impaired insulin signalling pathway results in a decreased Aβ-dependent hyperphosphorylation of the tau protein [257], thus suggesting a role of GSK3β in Aβ binding and in hyperphosphorylation of Tau.

1.3.3 Cholinergic hypothesis

This hypothesis, proposed more than 30 years ago, suggests that acetylcholine dysfunctions in brain neurons has a substantial role in cognitive decline, either in elderly subjects and in AD patients [253]. One of first considerations that led to this hypothesis was of an anatomical nature. The idea arose from the recognition of the high importance of cholinergic neurons in several functions connected to AD, such as consciousness, working memory, attention and a number of additional cognitive functions [191]. Many studies indeed, pointed out damages or anomalies of these ways in brains of elderly people and AD patients, suggesting a good correlation with the severity of cognitive decline. The main anomalies are the cortical deficiency of the

choline acetyl-transferase enzyme, responsible for the acetylcholine neurotransmitter synthesis [20], and the reduction of choline uptake. In consideration of this evidence, therapeutic approaches started with the purpose of compensating deficiencies of the cholinergic system through the administration of cholinergic agonists and inhibitors of acetylcholinesterase [253]. However, in the last decade, this hypothesis has been criticised because several studies showed that the acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) activity is not reduced in neocortex of patients affected by a mild form of AD.

1.3.4 Metabolic hypothesis

Alterations of the mitochondrial function came into researchers' attention in the study of several neurodegenerative diseases including AD. A negative correlation between aging, which is the major AD risk factor, and the reduced efficiency of the electronic transport chain (ETC) [182] suggested the idea that metabolic alterations leading to oxidative stress could be involved in the pathogenesis of the illness. Moreover, during lifespan, an increase in mitochondrial oxidative stress is usually observed [24]. In the 80s, metabolic defects in two enzymes of the Krebs cycle were reported in AD patients [79, 242] and in 1990, for the first time a decreased activity of the cytochrome oxidase enzyme in AD patients was described [189]. In AD, the mitochondrial dysfunction can damage the cell in two ways:

1. through a significant increase in the production and release of reactive oxygen species (ROS);

2. causing a depletion of energy.

Recently, many studies focused on the relationship between the soluble Aβ oligomers and mitochondrial enzymes. In 2010, it was reported that within mitochondria of hAPP transgenic mice synapses, both the activity of the complex IV (cytochrome c oxidase) and the ratio of respiratory control are decreased, while the oxidative stress (measured through hydrogen peroxide and 4-hydroxynonenal levels) is increased compared to wild-type mice [62]. According to the latest studies, this was due to the binding of Aβ1-42 with the subunit 1 of the enzyme cytochrome c oxidase. This interaction could explain the breakdown of complex IV and therefore the metabolic alterations found in the disease. However, there are several systemic alterations related to the AD that are still under investigation. Body weight, bone density, muscle mass, levels of insulin, oxygen consumption, are all examples of metabolic parameters that are frequently associated to alterations in cognitive performances and need to be evaluated in relation to AD pathogenesis.

1.4 Clinical features and symptomatology

According to the current criteria, AD is classified as a primary degenerative dementia, characterized by a progressive decline of the cognitive functions such as memory, thinking, judgment, language, problem solving. Additional deficits include personality changes and movement impairment. According to the age of onset of the disease, patients could be divided in two groups: Early-Onset Alzheimer's Disease (EOAD) forms and Late-Onset Alzheimer's Disease (LOAD) forms. EOADs represent less than 5% of all AD cases and show peculiar characteristics, such as age at onset $<$ of 65 years, positive family history and more aggressive course than LOAD [281]. Early-onset forms are caused by mutations in three specific genes: the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) genes. Almost all these mutations follow an autosomal dominant mendelian pattern of inheritance and affect the physiological processing of the APP inducing a shift towards the production of β-amyloid forms or affect the structural features of Aβ making it more prone to aggregation [14]. LOADs represent 90-95% of all AD cases, are mostly composed by sporadic forms and their etiology is mostly unknown. They are multifactorial forms in which both genetic predisposition and environmental factors contribute to the genesis of the disease [28]. Clinically, AD moves forward three main stages, even if there is no a clear separation between them and overlaps may occur. In the first stage, also referred as mild AD, symptoms occur almost exclusively as amnestic alterations of recent events, circumscribed to sporadic episodes of everyday life (ongoing memory). Other alterations may also appear at this phase, for example slight language deficits (word-finding impairment, difficulty in semantic codification, reduced speech fluency), executive functions difficulties (problem solving, information processing) and topographical and temporal disorientation [236, 258, 269]. In the middle stage, referred as moderate AD impairments become more significant and other dysfunctions appear. The clinical picture at this stage is generally characterized by temporal-spatial disorientation, language disturbances, attention deficits, apraxia, agnosia and behavioural changes such as apathy, aggression and alteration of the sleep patterns. In the late stage - severe AD - symptoms get worse: psycho-behavioural alterations such as acquired sociopathy or severe apathy, psychomotor agitation and irritability may occur. Patients completely or largely lose their personal autonomy, the ability to communicate, to control sphincters and swallowing and eventually are bedridden. However, as Dubois and colleagues have underlined, the Alzheimer's pathology cannot be considered only in relation to its clinical stages [63]. Indeed, convincing evidence provided by advances in the comprehension of AD pathogenesis, suggested that the pathophysiological process of AD begins years, if not decades, before the diagnosis of clinical dementia [172]. According to this view, a novel subdivision of AD in distinct stages has been proposed: Preclinical AD phase: this phase is characterized by the absence of clinical symptoms and signs

and can last a very long time (decades); Prodromal AD phase: this is the phase when the first clinical symptoms are detectable. They are not yet so evident at this stage to be classified as a dementia. AD dementia phase: it starts when the cognitive deficits appear consistent and meet criteria for dementia. Moreover, according to the recently revised diagnostic criteria [166], AD is clinically considered as "possible", "probable", and pathophysiologically proved or "definite", depending on the presence of core clinical criteria , disease biomarkers and neuropathological typical features. In consideration of the recent relevant achievements coming from pathogenic studies, scientific efforts are now ongoing in order to improve the detection of the early AD phases, i.e. preclinical and prodromal stages. The great challenge for the next future is the discovery of novel early biomarkers, the development of new diagnostic tools that allow recognizing the pre-symptomatic phases of the disease, and the design of efficient strategies for prevention and treatment for AD (Figure 1.2).

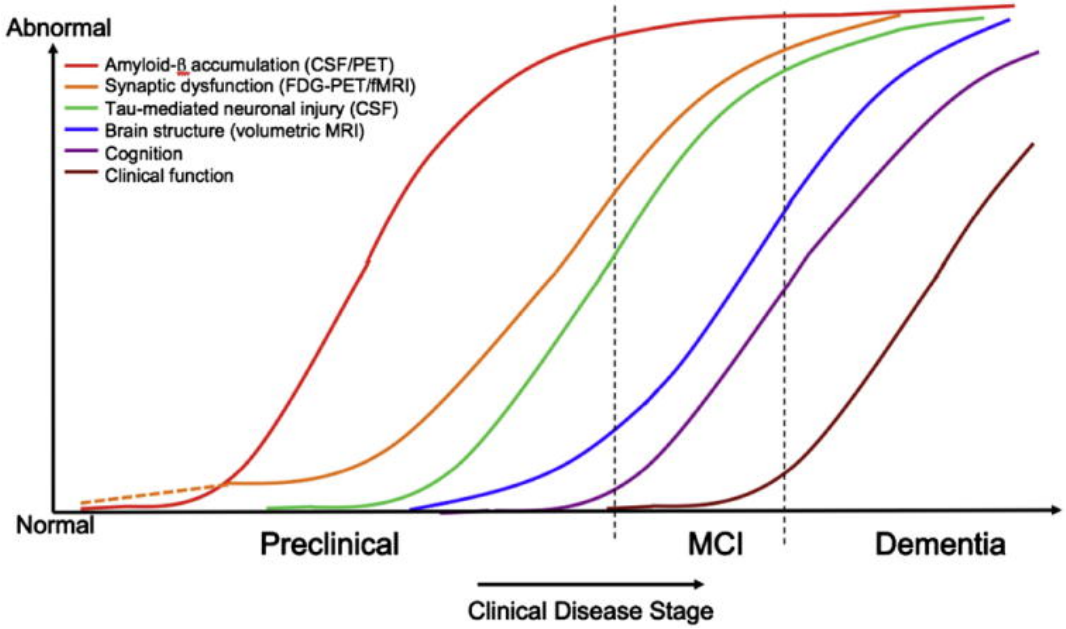

Fig. 1.2: Hypothetical model of dynamic biomarkers of the AD expanded to explicate the preclinical phase [243].

1.5 Diagnosis

Clinical diagnosis of AD is nowadays reached by the application of the new diagnostic criteria established by the National Institute of Aging and Alzheimer's Association (NIA–AA) [166, 2, 243]. The revision of previous criteria has led to several improvements in the diagnostic protocol and to the recognition of distinct phases along the course of the disease: a long pre-dementia stage was acknowledged; the mild cognitive impairment (MCI) was recognised as prodromal AD if amyloid and neurodegeneration biomarkers are present; biomarker evidence could be used to assign an high, intermediate, or low likelihood to the diagnosis of AD; atypical

presentations are acknowledged - even if the recognition of atypical clinical phenotypes is still deeply challenging - and memory impairment is no longer required for diagnosis if biomarker evidence is present.

1.5.1 Core CSF biomarkers

Primary CSF biomarkers for AD are: Aβ42 levels as indirect proof of cortical amyloid deposition; total tau (t-tau) and phosphorylated tau (p-tau) as indicators of downstream neuronal degeneration or injury [18]. These core CSF biomarkers are able to identify prodromal AD in the MCI stage with high diagnostic accuracy (sensitivity and specificity of 85-90%) [237, 262]. For these reasons, CSF biomarkers are helpful indicators that support neurologists during the diagnostic decision making.

1.5.2 Novel CSF biomarkers

The research on novel biomarkers is considered fundamental in AD. Additional measures of the pathogenic processes can be helpful to provide new insights into the molecular mechanisms of the illness. One example is offered by Aβ oligomers, the most toxic forms of Aβ responsible to the synaptic dysfunction [185]. In literature, an increase in CSF-Aβ oligomers has been reported in AD patients [113], but it often overlaps with control groups. Unfortunately, Aβ oligomers are difficult to detect in CSF, thus limiting their quantification by ELISA [283]. Recently however, Nakamura et al., unveil a sensitive and reproducible blood assay that can predict amyloid status with up to 90% accuracy. The test uses immunoprecipitation of Aβ peptides followed by mass spectrometry, and it works as well as existing CSF biomarkers to detect brain amyloid [175]. Another novel candidate biomarker is the dendritic protein neurogranin, a synaptic protein involved in long-term potentiation and memory consolidation [143]. High CSF levels of neurogranin prognosticate the progression of MCI patients towards AD and correlate with rapid cognitive decline [226]. On the other hand, presynaptic protein SNAP25 concentrations increase extensively during the prodromal stage [23]. Thus, synaptic biomarkers could be a link to both cognitive symptoms and therapeutic response.

1.5.3 Imaging

With the fast improvement of technology, imaging techniques have achieved a fundamental role in the diagnosis of AD. In patients with cognitive impairment is commonly used a visual scale rating of medial temporal atrophy as a diagnostic tool (e.g. hippocampal volumetry) [227, 228], MRI remains the gold standard for the assessment of vascular brain changes, such as white matter hyperintensities, lacunes,

and microbleeds [40, 15] and PET is useful during the early stage of the disease when the differential diagnosis is still wide and overlaps physiological ageing [2]. While a normal FDG PET theoretically excludes a diagnosis of neurodegenerative disease [190] a positive scan can suggests an AD diagnosis, if the pattern is temporo-parietal and posterior cingulate, and frontotemporal dementia if the pattern is anterior or asymmetric, or both [279]. One of the most promising new imaging marker for AD is PET with ligands for Aβ. Up to now, three ligands have passed the authorization for clinical use by the European Medicines Agency and the US Food and Drug Administration: florbetapir, florbetaben, and flutemetamol [110]. These ligands have very high accuracy in detecting cortical amyloidosis [30]. However, since brain amyloidosis is a necessary but not sufficient condition to diagnose AD, the diagnostic value of amyloid PET is more exclusionary than inclusionary. Fluorinated ligands for tau have also been developed, and they bind to tau fibrilles [261]. Moreover, tau ligands have shown a better correlation with hypometabolism and atrophy than does amyloid PET [184]. Combination of all the biomarkers described above — ie, hippocampal volumetry, FDG PET, amyloid PET, CSF Aβ42, t-tau, and p-tau, as suggested in the NIA-AA criteria — has good positive and negative predictive value in the discrimination of AD from normal ageing in patients with MCI.

1.6 Neuropathology

The two fundamental neuropathological hallmarks of AD are the extracellular deposition of Aβ peptides, which can accumulate in neuropils (plaques) or in blood vessel walls, and the formation of pathologic Tau protein within neurons as NFT, degenerative neurites around plaques or neuropil threads. Moreover, AD may be characterized by other neuropathological features that involve neurodegeneration, synaptic and neuronal loss, gliosis especially occurring in the cortical association areas, with consequent brain atrophy.

1.6.1 Macroscopic changes

The typical macroscopic alteration of the brain of AD patients is a symmetric cortical atrophy of the medial temporal lobes, with a concomitant dilatation of the lateral ventricles, especially in the temporal horns [233]. This morphological remodelling of the cortical structures can be identified in the primary stage of the disease through the magnetic resonance imaging (MRI) scan and represents one of the earliest criteria employed in the diagnosis of AD [58, 57]. The cortical atrophy is mainly linked to neuronal degeneration and synapse loss that involve the cortical association areas, the hippocampus and the entorhinal cortex, with a relative sparing of the subcortical structures like the cerebellum and the brainstem. Interestingly, synapses

loss may precede neuronal depletion, leading to a reduction of the synaptic density, and therefore, to the interconnection among the remaining neurons. This alteration can even be prevalent within specific cortical areas, indicating the synaptic density as a good parameter that correlates with cognitive decline in AD [52, 120, 254, 160]. This spatio-temporal neurodegeneration process is the key factor that induce the macroscopic alteration of the brain structure, as well as, the clinical picture of the disease.

1.6.2 Microscopic changes.

Three main microscopic alterations characterize the neuropathological picture of AD: the amyloid plaques, also called senile plaques, composed by extracellular accumulation of β-amyloid peptides (Aβ); the cerebral amyloid angiopathy (CAA), or congophilic angiopathy, due to the deposition of beta-amyloid peptides in the walls of blood vessels and abnormally modified forms of tau protein that accumulate intracellularly as paired helical filament (PHF)-tau and neurofibrillary tangles (NFTs) [268, 91, 92].

Senile Plaques

Senile plaques, are roundish extracellular structures with a 40-200 micrometres diameter, predominantly composed of insoluble deposits of β-amyloid peptide and many other protein [43], including apoE [176], components of the complement cascade [66], cytokines and dystrophic neurites [59, 89]. The plaque core is surrounded by reactive astroglia and microglia, leading to proposals that microglia may contribute to the formation of plaques [278] or may be engaged in phagocytosis of the amyloid fibers [136]. According to the morphological classification is possible distinct two types of plaques: an earlier form named diffuse plaques and a mature one called neuritic or dense-core plaques. The distinction is based on the affinity to the Congo red and Tioflavin-S staining, which highlight the β-sheet structural conformation. This categorization has a pathological relevance, because unlike the diffused Tioflavin-S negative plaques, neuritic plaques are associated with deleterious effects on the neuronal function, altering the neurite shape, promoting the synapses loss, activating astroglial and microglial cells [123, 199], up to the complete degeneration of neurons. The distribution pattern of amyloid plaque deposition is variable and not very predictable compared to that of neurofibrillary tangles. According to the Thal's stage hypothesis this pattern can be summarized in three phases: the first deposition phase involves the associative areas of the isocortex, the second phase involves the allocortex and limbic area (entorhinal cortex, hippocampal formation, amygdala, insular and cingulate cortex) and in the last phase, the subcortical areas

are affected [255]. Although the amyloid burden in the Alzheimer's brain is an unchallengeable data, its functional correlation with the development of the disease has always raised a great debate in the scientific community. First of all, diffuse amyloid plaques are physiologically present in the brains of older people, even if in small numbers. Secondly, they are not well correlated with clinical manifestations or cognitive impairment of patients. Therefore, amyloid plaques themselves are not directly responsible for the neuronal toxicity in AD but, as Selkoe and colleague have underlined, other toxic Abeta species, like the soluble oligomeric forms of Aβ, which precede the formation of senile plaques, are the most important players of synaptic and neuronal damage [231]. Although nowadays the key role in the neuronal toxicity of AD is mainly attributed to the oligomeric Aβ assemblies, the molecular mechanism through which this toxic effect leads the neurodegeneration remains still unclear.

Cerebral Amyloid Angiopathy (CAA)

Neuritic plaques are not the only type of deposit of β-amyloid in the brain of AD patients. Aβ can also accumulate in the walls of blood vessels. Beyond the location, these aggregates differ from senile plaques also in the content of Aβ isoforms: while the Aβ 1-42 tends to deposits in the brain parenchyma, the shorter and more soluble 1-40 isoform, preferentially accumulate in the interstices between the smooth muscle cells in the medial tunic of the vessels [121, 104]. This vascular deposition is usually visible in the cortical capillaries, in the small arterioles of the leptomeninges and brain parenchyma. According to the current view, the Aβ produced by neurons, is drained out along the perivascular interstitial fluid pathways of the brain parenchyma and leptomeninges where it can accumulate if their clearance is impaired [270, 271]. When an extensive accumulation takes place in vessels, spontaneous vascular breaks can occur leading to focal brain hemorrhages which can typically occur in combination with little ischemic events due to the thickening of the vessel walls. Although CAA is not a specific feature of AD, about 80% of AD patients show a mild degree of congophilic angiopathy at the autopsy examination. Interestingly, three independent longitudinal post-mortem studies have establish a positive relation between the asymptomatic CAA condition and the cognitive decline in AD [87, 178, 195].

Neurofibrillary tangles (NFTs)

Neurofibrillary tangles are filamentous structures mainly composed by the cytoskeletal tau protein, which accumulate inside neurons. Thanks to specific mechanisms of

phosphorylation/dephosphorylation, the tau protein plays a key role in the assembly, stabilization and functionality of microtubules. In pathological conditions, like AD, tau undergoes an abnormal process of hyperphosphorylation, which determines its detachment from microtubules and their consequent disassembling. Hyperphosphorylated tau begins to accumulate in the cytosol as non-fibrillary and non-helical inclusions called "pretangles" [142]. Subsequently, these structures can organize each other into PHF, rectilinear filaments (SF) (in dendrite) or in NFTs. Thanks to its sticky properties, tau can also bind other kind of proteins such as ubiquitin [192], cholinesterases [168] and β-amyloid peptide [116] contributing, in this way, to the formation of very complex insoluble structures. In addition to the destabilization of microtubules damage, these aggregates compromise the physiological functions of neurons, impairing the cellular morphology, the synaptic function and the axonal transport up to the death of cells. As a consequence, tau can be released by dead neurons (as proved by the increase of CSF-tau levels occurring in in AD [224]) and actively spreads across the brain areas. Recent studies in animal models have shown that intracerebral injection of misfolded tau protein can be internalized by cells [98, 97, 75, 280] and can propagate through interconnected brain regions [34, 31, 33, 148] while in vitro studies have demonstrated how tau can be released from several cell lines [133, 240, 201]. Overall these findings suggest that propagation of tau pathology is mediated by the release, uptake, and trafficking among interconnected neurons. Once internalized within cells, misfolded tau acts as a “seed” that recruits soluble endogenous tau into larger pathological conformations [130]. Importantly, this spatio-temporal tau spreading observed in brain of AD patients well correlates with patient cognitive decline, candidating phosphorylated tau as a good biomarker of the development of the AD [9, 22]. Despite this, it is still unclear whether the formation of NFTs is a necessary precursor of neuronal death in AD or represents a consequence to the neuronal damage. Furthermore, this type of pathological lesions can be also associated to other pathology such as FTD, FTDP and Pick disease (PD) [278]. Moreover, several genetic studies have shown how mutations in MAPT gene, encoding for tau protein, can cause FTD or FTDP [84], but do not lead to the AD. Probably the concomitant aberrant activity of the tau and Aβ proteins is necessary to the development of AD. In spite of that, the analysis tangles are an integral part of the neuropathological diagnosis of AD. Neurofibrillary tangles can be labelled in silver staining, with the Tioflavin-S fluorochrome or by immunohistochemistry with specific antibodies directed to the hyperphosphorylated tau protein. The pattern of distribution of NFTs in AD is, in most cases, stereotyped or otherwise predictable. The brain areas involved mainly are the hippocampus, the Amygdala and the second layer of the entorhinal cortex. Braak H. and Braak E. have in the past proposed a staging of the disease, still followed, which is based precisely on the distribution of tau deposits in the brain [21]. The current neuropathologic criteria for the diagnosis of AD are based on three parameters, “ABC”: Aβ/amyloid plaques (A), NFTs (B), and neuritic plaques (C) [117] (Table 1.1).

Aβ plaque score	NFT stage	Neuritic plaque score
A0 no Aβ or amyloid plaques	**B0** no NFTs	**C0** no neuritic plaques
A1 Thal phases 1 or 2	**B1** Braak stage I or II	**C1** CERAD score sparse
A2 Thal phase 3	**B2** Braak stage III or IV	**C2** CERAD score moderate
A3 Thal phases 4 or 5	**B3** Braak stage V or VI	**C3** CERAD score frequent

Tab. 1.1: Schematic classification of the main neuropathologiacal criteria adapted for the diagnosis of AD.

Aβ deposits can be at the center of a cluster of dystrophic neurites that frequently, but not always, have phospho-tau immunoreactivity; these are a subset of senile plaques called neuritic plaques. neuritic plaques have been considered to be most closely associated with neuronal injury. Indeed, neuritic plaques are characterized by the occurrence of dystrophic neurites, greater local synapse loss and glial activation. NFTs are commonly observed in limbic regions in the early AD stages but, along with the disease, progression, can be also found in other brain regions, such as association cortex, some subcortical nuclei, and even some brainstem regions where their formation may proceed that in limbic structures. Common classification criteria for NFTs apply the six stages categorization proposed by Braak and Braak: no NFTs, Braak stages I/II with NFTs predominantly in entorhinal cortex and closely related areas, stages III/IV with NFTs more abundant in hippocampus and amygdala while extending slightly into association cortex, and stages V/VI with NFTs widely distributed throughout the neocortex and ultimately involving primary motor and sensory areas. The 2012 criteria adopted a previously developed Consortium to Establish a Registry for AD (CERAD) neuritic plaque scoring system, which ranks the density of neuritic plaques identified histochemically in several regions of neocortex.

1.7 AD genetics

1.7.1 EOAD

More than 200 mutations of PSEN1, PSEN2, and APP genes have been shown to cause EOAD [115]. As carriers of mutations in these genes will almost cer¬tainly develop AD, they provide a unique group to characterize the trajectory of preclinical AD changes in relationship to their family's estimated age at clinical onset [25]. EOAD differs from the more common, late - onset form of AD in several respects - for example, by a gen¬erally younger age at clinical onset, and for the over - representation of atypical or unusual AD phenotypes, which are more common among EOAD cases. However, the clinical heterogeneity within the sporadic group is probably underestimated.

1.7.2 LOAD

Late-onset Alzheimer is the most common form of the disease and is defined by onset age 65 years. Whereas EO-FAD is characterized by classic Mendelian inheritance usually in an autosomal-dominant manner, for LOAD, we observe a genetically complex pattern of inheritance in which genetic risk factors work together with environmental factors and life exposure events to determine lifetime risk for AD. Consequently, it is much more difficult to reliably identify novel LOAD loci [252]. The major genetic risk factor for LOAD is the APOE, a polymorphic gene with three distinct alleles named $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$. This last isoform, is associated to an higher risk to develop the illness [39, 248]. Indeed, as proved by several genome wide association studies (GWAS), $\epsilon 4$ heterozygous carriers show a 2-3 fold increased risk to develop AD while homozygous carriers have about 12-fold higher risk compared to $\epsilon 3$ carriers [16, 70]. Apo$\epsilon 4$ protein has various effects on AD pathogenesis, for example it can interfere with the Aβ clearance in the brain [26], and can be also processed into neurotoxic fragments. Moreover, it has been seen that APOE4 transgenic mice show a degeneration of brain blood vessels, resulting in the leakage of the blood-brain barrier and in an Aβ-independent neurodegeneration [158]. Up to now, GWAS have identified more than 20 genetic loci associated with AD risk. The newly identified genes are implicated in the immune system and inflammatory response pathways, as well as in the cholesterol and lipid metabolism, and in the endosomal-vesicle recycling [93]. Among them, the best validated is TREM2, a microglia receptor involved in Aβ clearance [286]. New genome-sequencing technologies are continuously to identify rare mutations able to modulate substantially the risk to develop AD [94, 44](Figure 1.3). Studies of individuals with inherited AD can provide insights into cognitive and biomarker changes that precede clinical manifestation of the disease, and are suitable candidates for ongoing monitoring and early-intervention strategies. Unfortunately, all the diagnostic techniques described above are able to detect AD in the symptomatic stage of the illness - and therefore when the damage is already irreversible - but often are insufficient to recognize atypical phenotypes of the disease. These limitations influence negatively the therapeutic approach, limiting its indication to the treatment of symptoms but not of the disease. Considering that, there is an urgent need to find novel specific biomarkers for the asymptomatic stage of the AD, that allow a very early diagnosis and improve the successful chances of the therapeutic interventions.

1.8 Therapeutic strategies

The lack of a full and clear knowledge of pathogenesis and the difficulties in achieving an early diagnosis make the AD an untreatable pathology. The only available

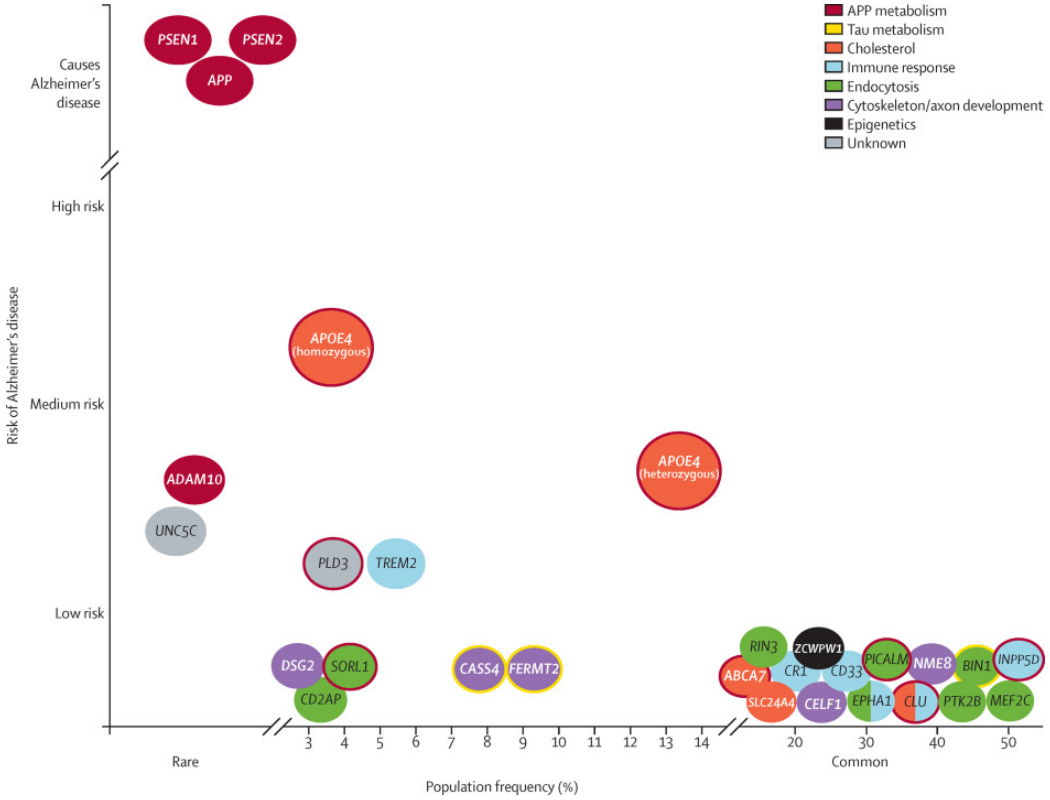

Fig. 1.3: Schematic overview of genes linked to Alzheimer's disease. The colours in the key show the pathways in which these genes are implicated. The interior colours provide further information on what functions the genes have. When there are two colours, the gene might have functional roles in two different biological pathways. Many of the genes have been related to APP processing or trafficking suggesting the central importance of APP metabolism in Alzheimer's disease. The figure was adapted with permission from Karch et al, 2015.

therapies are symptomatic treatments aimed to enhance cognitive performance and to control neuropsychiatric symptoms. A number of disease-modifying therapies (DMTs), based on the use of drugs that prevent, delay, or slow the disease progression, are under evaluation. According to a recent review of Jeffrey Cummings, that looks at the clinical active trials reported on the clinicaltrials.gov at January 2018 [45], there are 112 agents currently in the pipeline for the Alzheimer's disease therapy: 23 agents are in 25 trials in phase I 63 agents are in 75 trials in phase II 26 agents are in 35 trials in phase III

Of all, 63% are DMTs, 22% are cognitive enhancers, 12% are aimed at neuropsychiatric and behavioural changes and 2% have undisclosed mechanism of action (MOAs) (Figure 1.4).

According to the previous study of Cummings, Morstorf and Zhong, whose reviewed the Alzheimer's clinical trials from 2002 up to 2012, the success rate of these interventions is around to 0.4% (99.6% failure) [46]. Considering that, phase III trials require high time and money investments, their results lead to question if research is going towards the right direction. It is always clearer that AD is a

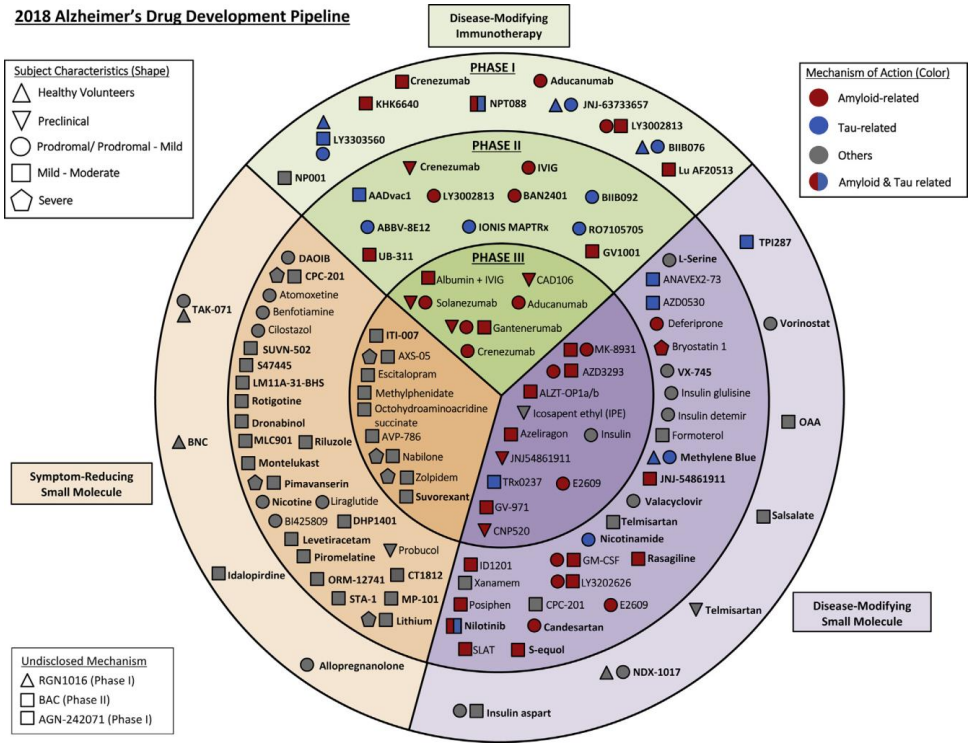

Fig. 1.4: Agents in clinical trials for treatment of Alzheimer's disease in 2018 (from clinicaltrials.gov accessed January 30, 2018

very complex and multifactorial disorder and its clinical and biological variability is gaining much more attention, and thus it should be considered in therapeutic approaches. In this sense, the AD heterogeneity is one of the main underestimated factors that may contribute to the high amount of trial failure and it should be taken into consideration in the design of therapeutic approaches to AD.

1.9 Phenotypic heterogeneity of Alzheimer's disease

AD is traditionally considered as a single clinico-pathological disorder characterized by progressive loss of memory and behavioural impairment that ultimately leads to the complete loss of autonomy and self-care. However, increasing data on clinical, genetic, and neuropathological heterogeneity of the disease suggest that AD should not be considered as a unitary pathology. Despite this, most of the current clinical trials do not discern between disease subtypes, introducing biases in the studies of efficacy of therapeutic compounds. Thus, it is crucial to characterize in details all the phenotypes enclosed in AD and categorize patients according to their clinical and biological features.

1.9.1 Clinical heterogeneity

The most frequent form of AD presents with an amnestic picture characterized by episodic memory deficits related to dysfunctions of the hippocampus and alterations in the mesial temporal lobe circuitry [97]. However, AD can also display atypical clinical phenotypes, such as:

1. The “visual” variant, characterized by visuoperceptual and visuospatial alterations;

2. The “logopenic aphasia”, defined by primary impairment of the language;

3. The “frontal” variant, whose main features are the impairment of executive cognitive functions andand the presence of releavnt behavioral abnormalities [265].

These atypical forms of AD are caused by selective dysfunction of brain areas, which may be differentially involved in the spreading of the pathogenic Aβ and p-tau proteins in the brain [265, 230, 197]. Unfortunately, mechanisms that make some brain regions more susceptible than others to Alzheimer’s pathology remain largely unknown. A very recent study from Michel J Grothe and colleagues tried to give an explanation by comparing the neuroimaging patterns of amyloid deposition/neurodegeneration with the regional gene expression profiles of the human brain from AD patients. They noticed a positive correlation between the regional APP expression levels and the amyloid deposition, whereas no correlations were found with the neurodegeneration process. The opposite pattern was instead observed for MAPT, since tau-related neuropathological changes correlate with neurodegenerative changes and synaptic dysfunctions. The regional gene expression analysis revealed also which is the gene sets deregulated in amyloid-vulnerable regions as well as in neurodegeneration-vulnerable regions. In the first case, genes involved in protein synthesis and mitochondrial respiration are lower expressed, while in the second case genes implicated in neuronal plasticity, neurite outgrowth, synaptic contact, intracellular signalling cascade and proteoglycan metabolism are higher expressed. These findings suggested that the selective susceptibility of different brain regions to AD pathology relies on specific biochemical pathways and that these, very differ among amyloid accumulation and neurodegeneration brain regions [90]. Natalie Ryan and colleagues reported on the Lancet Neurology in 2016 the results of a study describing heterogeneous cognitive symptoms and neurological features in a large series of EOAD patients. They report a large case of 213 patients mutated in PSEN1 or APP, taking into consideration non-cognitive symptoms such as myoclonus, seizures and pyramidal, extrapyramidal and cerebellar motor dysfunctions. Results

show a clear stratification of PSEN1 carriers, which exhibit an earlier age at onset, and a more common atypical presentation of symptoms. In addition, individuals with myoclonus (47% with PSEN1 and 33% with APP mutations) were significantly more likely to develop seizures [220]. The overall data suggest how important is the clinical heterogeneity of AD patients. However, while such variability is well known and accepted for EOAD, the heterogeneity for the sporadic forms remains underestimated and needs to be supported by studies that take into consideration also biological changes both at cellular and molecular level, which may underlie the clinical variability of the disease. Understanding the molecular basis of such heterogeneity may offer grounds to early identify disease subtypes and to cluster AD patients according to their clinical profile. The early recognition of atypical forms of the disease may help to improve the diagnostic protocols for AD and may also provide useful information about the progression of the disease. Finally, the knowledge of the molecular mechanisms underlying the phenotypic variability of AD may help to design more personalized treatments for patients.

1.9.2 Biological heterogeneity

Emerging evidence supports the idea of a biological variability among AD patients. Although the concept of heterogeneity is relatively new, a first level of variability is already appreciable looking at one of the two main molecular players in AD pathology: amyloid-beta peptide. Pathogenic forms of β-amyloid protein are the result of an altered cleavage of the longer Amyloid Precursor Protein (APP) by means of groups of endogenous proteases, called secretase. APP can undergo two major proteolytic processing pathways (see paragraph on AD pathogenesis). In particular, the variable cleavage of APP by the enzymes involved in the amyloidogenic pathway, i.e. β- and γ-secretases, leads to the release of distinct Aβ peptides of 37, 38, 39, 40 or 42 amino acid in length [203]. This pathway contributes to the heterogeneity of the Aβ peptides accumulating in brain tissue and may confer to the mixtures of Aβ isoforms a variable propensity to aggregate and generate oligomers, fibrils and amyloid deposits. Other two factors contribute to the generation of distinct isoforms of the Aβ peptide. The first one is the N- and C-terminal truncation cleavage: Aβ can undergo digestion by several proteases like IDE, NEP and MMPs responsible of the truncated form of the peptide [263]. Up to now, more than ten N-terminally extended (NTE) Aβ fragments have been characterized in CSF [204, 275] and in vitro studies showed that the deletion of the N-terminus rises the capacity of Aβ to aggregate [199]. The second one, includes post-translational modifications (PTMs) such as oxidation, phosphorylation, Nitric-oxide-caused modifications, Glycosylations, Pyroglutamylation, Isomerization and Racemization [141]. Among these, pyroglutamylation was recognized as one of the most important events of amyloid cascade hypothesis related to the AD pathogenesis.

PTM	Site	Description	References
Oxidation	Met35	impedes the formation of Aβ protofibrils and fibrils from monomers	N“aslund J. et al., 1996. Palmblad M. et al., 2002. Hou L. et al., 2002.
Phosphorylation	Ser8, Ser26, Tyr10	increases the formation of oligomeric Aβ aggregates that represent nuclei for fibrillization, serine 8-phosphorylated Aβ is resistant to degradation by insulin degrading enzyme	Milton NG. 2001. Kumar S. et al., 2011. Kumar S. et al., 2012. Kumar S. et al., 2013.
Nitric-oxide-caused modifications	Tyr10	dityrosine-coupled Aβ stabilize Aβ dimers. Nitrated Aβ was able to initiate plaque formation and suppress Hippocampal long-term potentiation in APP/PS1 mice.	Castegna A. et al., 2003. Butterfield DA. et a., 2007. Kummer MP. et al., 2011. Al-Hilaly YK. et al., 2013.
Glycosylations	Tyr10	Glycosylation occurred on Aβ1–15/16/17/18/19/20, Aβ3–15, Aβ4–15, Aβ4–17, and Aβ5–17 peptides, but not in Aβ1-40/42.	Halim A. et al., 2011.
Pyroglutamylation	Glu3, Asp11	Increased propensity to aggregate and to form β-sheets in vitro	Mori H. et al., 1992. N“aslund J. et al., 1994. N“aslund J. et al., 2006. Tekirian TL. et al., 1999. Youssef I. et al., 2008.
Isomerization	Asp1, Asp7, Asp23	increase the tendency form β-pleated sheets, aggregates and enhanced insolubility and resistance to enzymatic degradation. isoaspartate-7 could be an indicator of plaque age.	Fabian H. et al., 1994. Shimizu T. et al., 2002. Fukuda H. et al., 1999. Fonseca MI. et al., 1999.
Racemization	Ser8, Ser26, Asp1, Asp7, Asp23	D-Ser26-Aβ1–40 possesses a stronger tendency to form fibrils	Tomiyama T. et al., 1994. Kubo T. et al., 2003.

Tab. 1.2: Schematic illustration of the main Post translational modifications (PTMs) related to Aβ peptides involved in the Alzheimer's pathology.

Additional factors potentially contributing to AD heterogeneity come from the second pathogenic peptide involved in the pathogenesis of the disease: tau protein. Adult human brain expresses six isoforms of tau protein, differ each other by the presence or absence of one or two inserts (29 or 58 amino acids) in the N-terminal part and by the presence of either three or four MT-binding repeats (R) in the C-terminal region. All the isoforms are the result of an alternative splicing of the single MAPT gene [83]. Specifically, the alternative splicing of the exon 10 leads to the expression of either 4R-tau or 3R-tau [82, 8]. The balance between the two isoforms seems to be important in pathological conditions: while normal adult human brain expresses equal levels of 3R-tau and 4R-tau [81, 137] altered 3R/4R-tau ratios have been observed in several tauopathies [84, 61]. Moreover, functional differences were demonstrated between 4R and 3R tau. For example, it has been shown that 4R tau isoforms are more efficient at promoting microtubule assembly than 3R tau isoforms [99]. Like Aβ, also Tau can undergo post-translational modifications, include phosphorylation, glycosylation, ubiquitination, glycation, polyamination, nitration, truncation, and aggregation. Certainly, phosphorylation is the most known PTM involved in AD. For the first time, in 1984 Lindwall and Cole demonstrated that phosphorylation of tau destabilizes the assembly of microtubules [152]. Together with the evidence of 3- to 4-fold increase in phospho-tau levels in Alzheimer's brain compared to normal human brains [139, 132], P-tau added another important piece to the illness picture. To date, more than 40 phosphorylation sites have been identified in tau protein isolated from AD brain [85] and P-tau is use as a core biomarker in the diagnosis. To date, only few no studies have explored the potential correlation between tau –related pathology and AD clinical/neuropathological heterogeneity. Among them it is worth to mention a study by Janocko NJ and colleagues, who proposed in 2012 a novel classification of AD - based on the relative density of neurofibrillary tangles (NFTs) in the hippocampus and association cortices - into 3 subtypes: typical AD, hippocampal-sparing AD (HpSp AD), and limbic-predominant AD (LP AD), opening the way to further studies on the role of tau as molecular determinant of AD phenotypic variability [125].

1.9.3 Prion like hypothesis

The presence of different Aβ peptides implicates the formation of polymorphic aggregates distinguishable for their morphology and biochemical composition [68, 150]. Similarities with prion diseases suggest that the phenotypic heterogeneity of AD may depend on conformational modifications of the Aβ peptide [214, 38]. Analogously to what happens for prions, the misfolding of Aβ could induce the formation of pathological conformers prone to aggregate in toxic soluble forms and insoluble assemblies. Experimentally, cerebral amyloidosis can be exogenously induced by intracerebral inoculation of brain extracts containing aggregated Aβ

seeds. These seeds of Aβ are able to accelerate the amyloidogenic process only in transgenic animals that over-express the APP, while they do not affect the non-transgenic control mice. The induction of specific lesion profiles is led by structural and biochemical nature of Aβ seeds, as well as by features of the host (Figure 1.5) [129]. These evidences seem to suggest that the concept of prion-like conformational strains can also be applied to cerebral β-amyloidosis, reinforcing the hypothesis of the prion-like misfolding mechanism of Aβ [106]. Recent studies revealed that both soluble and insoluble forms have the capacity to induce amyloidosis in vivo but fragmentation of the large insoluble seeds into smaller and more soluble multimeric structures increase the seeding ability and the spreading of toxic aggregates [145]. In addition, intraperitoneal injection of Aβ-rich brain extract into a transgenic mouse for the APP gene is able to induce β-amyloidosis in the brain after a prolonged period of incubation [67]. Remarkably, induction and spreading of protein aggregates through a prion-like mechanism, seems to be a common feature of neurodegenerative diseases, such as Parkinson's disease, Amyotrophic Lateral Sclerosis, Huntington and others. At present, it is not known whether seeds of different types can lead to the genesis of distinct phenotypes of the same disease.

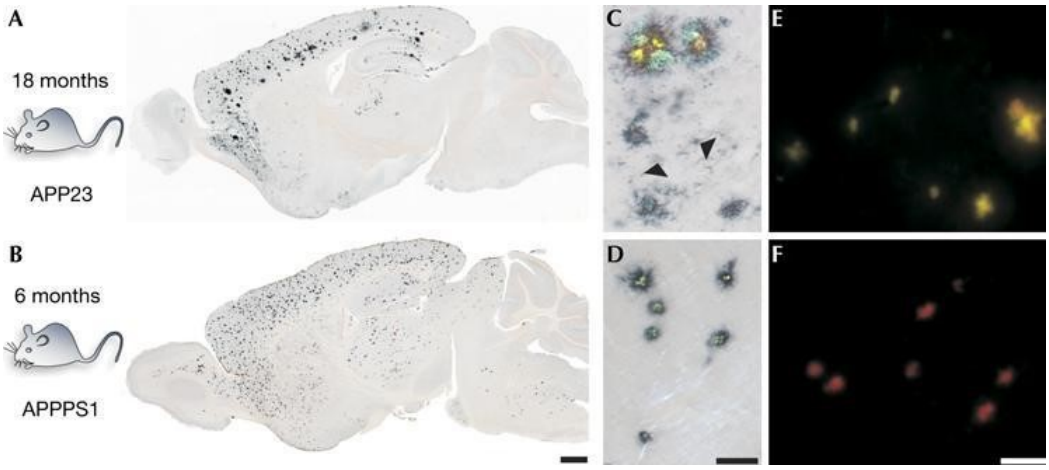

Fig. 1.5: Aβ deposits in APP23 and APPPS1 mice differ in morphology, spectral properties and Aβ40/42 ratio. (A,B) Aβ-immunostaining and Congo red staining of an 18-month-old APP23 and 6-month-old APPPS1 mouse. (C,D) Higher magnification of the hippocampal plaques. Note the rather large congophilic (yellow–green birefringence) plaques and diffuse (arrowheads) Aβ deposits in APP23 mice (C) in comparison with the smaller, compact and congophilic plaques in APPPS1 mice (D). (E,F) τPTAA staining of the hippocampal plaques. Note the shift to yellow–green colours of the Aβ deposits in the 18-month-old APP23 mice (E) and the reddish colour of the deposits in the 6-month-old APPPS1 mice (F). Scale bars, 500μm (B), 100μm (D), 50μm (F).

1.10 Neuroinflammation

The term "inflammation" indicates a response of the nervous tissue against a potential harmful stimulus. This process can be promoted by a variety of cues, including infections, traumatic brain injuries, toxic metabolites and autoimmunity. Although it is frequently associated to a negative meaning, inflammation is also a protective

reaction, able to recall and activate the Immune System (IS) against the damage. When this process does not find an end, inflammation may become chronic or permanent and therefore, detrimental. The entire inflammatory reaction has been well characterized in the whole organism, but remain largely unclear in the Central Nervous System (CNS) where it is usually referred as neuroinflammation. Indeed, until few years ago, the brain was considered an "immune-privileged" organ without any interactions with the immune system [167, 274]. Several considerations led to this conclusion:

- Successful transplantation studies of tumoral [174] and fetal [276] tissues without rejection suggest the immunological unicity of the brain;
- The absence of lymphocytes production sites and lymphatic system, confirmed the separation between CNS and IS;
- CNS lacks of antigen presenting cells (APC). APCs are the mediator between the innate and the adaptive immunity. Therefore, indicating no communication between CNS and IS;
- The existence of the blood brain barrier (BBB) denoted a high grade of isolation from the rest of organs.

Recently, the idea of "Immune-privilege" has been reviewed. In 2015, Dr. Antoine Louveau and colleagues discovered for the first time the presence of a neuronal lymphatic system in mice [155]. Furthermore, new findings revealed the presence of Toll-like receptors (TLRs) and related signalling proteins on the microglial surface [134]. These proteins belong to the pattern recognition receptors (PRRs) family and play a crucial role in the proper function of the innate immune system. They are able to recognize an array of endogenous molecules termed danger-associated molecular patterns (DAMPs) and exogenous epitopes called pathogen-associated molecular patterns (PAMPs). There are evidences that activated microglia express MHCII, acting as APCs [138]. Eventually, under inflammatory conditions, the BBB can undergo leakage allowing the infiltration of lymphocytes. All these findings highlighted an existing relation between the CNS and IS, leading scientists to reconsider the role of inflammation in neurological disorders. AD for example, like almost all the neurodegenerative diseases, includes neuroinflammatory reactions. Indeed, thanks to the PRRs receptors, microglia and astroglia are able to bind misfolded and aggregated proteins triggering the innate immune response. In turn, the immune cells, including microglia, release inflammatory mediators that can contribute to disease progression and severity. If neuroinflammation is a cause or a consequence, protective or detrimental in AD has still to be proved. Certainly, it plays a pivotal role in the development of the illness and thus it cannot be ignored. In the AD, we

talk about neuroinflammation by referring to a set of cellular and molecular players that together contribute to the development of the inflammatory state in the nervous tissue. They are mainly four:

- The Blood brain barrier (BBB);
- Immune cells;
- Glial cells;
- Microenvironment.

1.10.1 The blood brain barrier

AD is associated with alterations of the neurovascular unit, induced by the synergic effects of soluble Aβ oligomers and vascular Aβ deposits [118, 238]. These pathological changes can compromise the cerebral blood flow rate and impair the functional hyperemia [194]. On the other hand, a minor blood afflux involves a minor oxygen supply, inducing a state of chronic hypoxia. Hypoxia, is able to improve the amyloidogenic pathway of the APP [287] and, together with the prominent inflammation of the BBB and the increased accumulation of Aβ, increases the overexpression of the receptor for advanced glycation end products (RAGE) that transports Aβ in the brain parenchyma across the BBB [51].

1.10.2 Immune system cells

Under physiological conditions CNS strictly regulates the passage of the immune cells across the BBB. Therefore, normally these cells are restricted to the perivascular and subarachnoid spaces and only rarely they enter the brain parenchyma [1]. Under the inflammatory state, the signalling pathway of chemokines recalls immune cells from the periphery, makes the mechanism of infiltration across the BBB became faster and stronger, and increase the number of immune cells in the brain parenchyma [95]. In AD, only few immune cells are demonstrated to actively participate in the neuroinflammation response: for examples even though peripheral blood mononuclear cells (PBMCs) and macrophages cross the BBB, it has been shown that they do not take part in the clearance of amyloid plaques [86]. The only direct contribute seems to come from Treg and Th17 cells. Treg are specific subpopulation of T lymphocyte able to modulate the balance between inflammation and immune tolerance. Saresella and colleagues showed that the development of AD is associated with lower quantities of circulating Treg lymphocytes and, in particular,

with reduced percentages of PD1neg Treg cells. The minor presence of this specific T cell population was linked to an increased amyloid-β-stimulated-T cells proliferation and a reduced ability of Treg to suppress such proliferation. These results support the hypothesis of an inflammatory origin of AD and indicate Treg lymphocytes as key players in the neuroinflammation associated to AD [225]. Interestingly, Zhang and colleagues hypothesized that Th17 cells are directly responsible for neuronal cell death through the interaction of transmembrane proteins Fas and FasL in aβ1-42-induced AD model of rats [286]. On the other hand, B cells are indirectly involved in AD neuroinflammation through the production of autoantibodies. A vast part of autoantibodies are believed to be produced in response to the toxic aggregated forms of Aβ, including oligomers and protofibrils both in periphery and in CNS [60, 250]. According to these evidences, therapeutic approaches based on the administration of antibodies against Aβ peptides were proposed.

1.10.3 Glial cells

As mentioned above, CNS is not more considered an immune-privileged organ but it actively participates with the IS to the neuroinflammation process. Glial cells are the most involved components in this reaction. They include oligodendrocytes, astrocytes, microglia and ependymal cells, and contribute to maintain the homeostasis of the CNS providing support and protection to neurons. The importance of the glial cells in the AD lies in their ability to activate themselves in the early stages of disease before the Aβ deposition and therefore before damages become irreparable [141]. Astrocytes have different roles, for example they possess both structural and metabolic functions, participate in the formation of the BBB, modulate the synaptic transmission and promote the recovery of the neural tissue after injury. In pathological conditions, such as AD, astrocytes become reactive but without losing the organization of their functional domains and without forming any brain scars. The reactive astrogliosis is initially marked by morphological changes like the atrophy of the cell body and its ramifications. This modification has been observed in vivo in the early stages of the disease, and it may reflect functional alterations in the synaptic transmission thereby contributing to cognitive deficits [183, 285, 140, 13]. Moreover, in the later stages of AD, astroglial atrophy proceeds with a clear spatio-temporal progression that raise from the entorhinal cortex up to the astrocytes located distantly from senile plaques. Astrocytes are also involved in the clearance of Aβ in vivo [282]. When exposed to Aβ peptides, they extracellularly secrete several proteases, such as NEP, insulin degrading enzyme (IDE), endothelin-converting enzyme-2 (ECE2), and angiotensin-converting enzyme-1 (ACE1) that are able to degrade Aβ peptides [221, 198]. Eventually, astrocytes act in the clearance of soluble Aβ from the parenchyma, through paravenous drainage [119]. It has been observed that this pathway relies on the astrocytic water channel aquaporin-4.

Indeed, its deletion results in a strong decrease of the Aβ clearance through this pathway. Microglia comprises a group of branched shape cells which are considered resident phagocytes of the CNS and account from 0.5% to 16.6% of all human brain cells. These cells are ubiquitously distributed within the brain but with significant regional differences [171]. Using their motile processes, they dynamically survey the surrounding environment for the presence of pathogens or cellular debris [211] and, in parallel, they contribute to protection, maintenance and remodeling of synapses [126]. Once activated by pathological inputs, microglia extend their processes at the site of injury, migrate to the lesion, and initiate an innate immune response. The first step requires the identification of detrimental targets (DAMPs or PAMPs) through ligation with the surface receptors. This bind induces morphological and functional changes leading microglia to the activated state: branches become shorten soma hypertrophic and microglia become able to engulf and digest the target. Simultaneously, microglia starts to secrete soluble factors including pro and antiinflammatory cytokines, chemokines, caspases, prostanoid and proteins of the complement system. These factors can act in an autocrine or paracrine way on the nearby microglial or astroglial cells and in an endocrine manner by recruiting cells of the immune system from the periphery of the organism. In AD, microglia are able to bind both the soluble Aβ oligomers and Aβ fibrils through scavenger receptor A1, CD36, CD14, α6β1 integrin, CD47 and toll like receptors (TLR2, TLR4, TLR6 and TLR9) [10, 188, 245, 154]. The binding of Aβ with these receptors results in activation of microglia that starts to produce proinflammatory cytokines and chemokines. At the same time activated microglia actively participate to amyloid clearance: microglial cells secrete several extracellular proteases, like NEP and IDE that are able to degrade soluble Aβ [149]. On the other hand, the Aβ fibrils, which are largely resistant to enzymatic degradation, are engulfed by phagocytosis. Inefficient clearance of Aβ has been identified as one of the major pathogenic pathways involved in the illness [164]. To support this hypothesis a recent study identified a mutation in the extracellular domain of TREM2, which induce a higher risk of AD in the similar extent as apoEϵ4 [94]. Other similar studies reported significant associations between four TREM2 variants and AD risk. TREM2 is highly expressed by microglia [74, 111] and reactive astrocytes surrounding plaques and mediates phagocytic clearance of Aβ [264]. Interestingly, some data indicate that in the aging CNS of mice, rats and primates, microglia show enhanced sensitivity to inflammatory stimuli [193], similar to that seen in microglia in brains with ongoing neurodegeneration. This phenomenon has been named priming and may be caused by microglial senescence representing an explicative element of the “time factor” for age-related neurodegenerative disease [247]. Microglial activation offers one of the best examples of heterogeneity observable during the neuroinflammation process. Based on macrophage classification, microglia was categorised as a classic proinflammatory (M1) phenotype associated with expression of proinflammatory cytokines and cytotoxic genes and a non-inflammatory (M2) phenotype associated with phagocytosis and releasing of

protective and trophic factors. However, the M1/M2 dichotomy represents just an oversimplified idea of two extreme activation states. The status of microglia in vivo, is much more complicated than that in vitro and may include a spectrum of different but overlapping functional phenotypes (Figure 1.6). For example, a growing body of evidence identified several subpopulation in M2 phenotype, named as M2a, M2b, M2c and Mox, each with distinct physiological functions in vitro [173, 47]. However, the roles of these subpopulations in vivo have not yet been defined. It is important to underline that M1 and M2 phenotypes are not beneficial or detrimental for their own but depend on the circumstance; In the APP/PS1 AD mouse model, for example, the transgenic expression of IL-1β led to robust neuroinflammation and a reduction of amyloid plaque pathology [235, 77]. The reason why this process became detrimental it is because does not find a resolution. Indeed, above all in AD, the inflammatory reaction is sterile, as it involves the very same receptors but no living pathogens. Under normal circumstances, such a reaction quickly resolves pathology with an immediate benefit to the nearby environment. In AD however, several mechanisms include the concomitant formation of Aβ and positive feedback loops between inflammation and APP processing compromise resolution of inflammation.

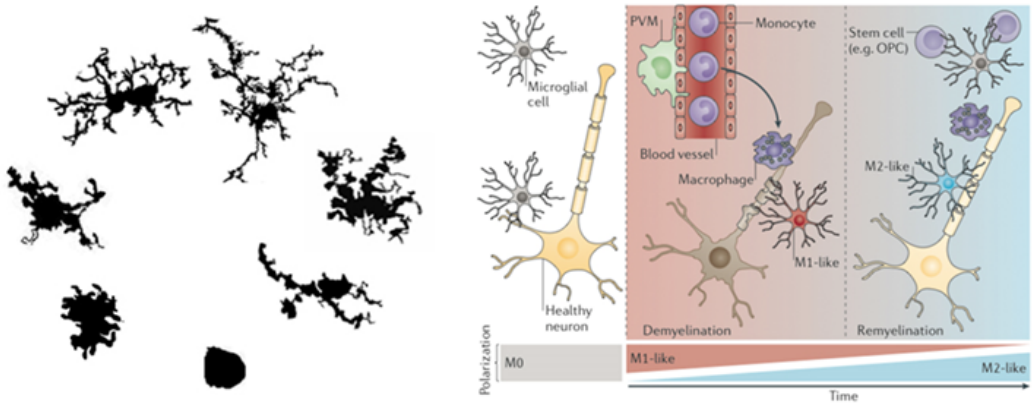

Fig. 1.6: Schematic representation of microglia. (a) Morphological changes from highly ramified to completely lacking processes. The transition can be very rapid or microglia can remain quiescent (M0) for years. (b) Functional simplification of the M1 and M2 phenotypes. Neuronal dysfunction or damage can activate microglia to produce pro-inflammatory cytokines, this state is called M1. Over time, depending on the type of brain injury or environmental factors, microglia may acquire an anti-inflammatory phenotype, known as M2, remove debris and promote regeneration. The activation states of microglia and macrophages can mix and switch [207].

1.10.4 Microenvironment

The microenvironment defines a functionally specialized and isolated area characterized by factors of both cellular or molecular nature. Specifically, the neuroinflammatory microenvironment is mainly composed by microglia, astroglia, as well as by immune cells and a long number of signalling molecules like cytokines, chemokines, caspases and protein of the complement cascade, secreted by them. Depending on the effect that these factors have in that specific context and in relation to the others,

they can be classified as pro- or anti-inflammatory agents. In AD several findings indicate the presence of a pro-inflammatory microenvironment: tumor necrosis factor-α (TNF-α) and interleukin (IL) -6, two of the major factors promoting the acute phase of the inflammatory response, have been found increased respectively in the serum and in brain tissue [11, 12] of AD patients compared to age-matched controls. Together with other findings which include the activated microglia surrounding senile plaques [102], the localization of T cells within Aβ deposits to, and the presence of reactive astrocytes in AD brains [277, 122], the data on TNF-α and IL suggest a strong involvement of the proinflammatory microenvironment in AD pathology.

2

Identification and characterization of AD molecular profiles based on the content of Aβ peptides in brains from AD patients with different clinical and neuropathological phenotypes: Molecular subtypes of Alzheimer's disease

2.1 Aims of the study

AD is a heterogeneous and complex disorder characterized by cognitive, behavioural and neuropathological alterations that lead patients to death, since no effective therapies are available. The heterogeneity of the disease is further complicated by the lack of valid biomarkers which can be usefully employed for the early recognition of atypical forms of AD, and has a likely negative impact on the effectiveness of therapeutic strategies, contributing to the failure of the therapeutic interventions tested until now. The definition of the molecular mechanisms which are responsible of the phenotypic variability of AD is an urgent need. Indeed, the identification of distinct phenotypes and the stratification of AD patients into different subgroups, even before the clinical onset of the disease, could help to anticipate the disease progression and to design tailored and more effective therapeutic strategies for each disease phenotype. According to the preliminary data produced by the Neuropathology Unit of the Foundation IRCCS "C. Besta" Institute, we found that distinct mixtures of Aβ peptides including the C- and N-terminal forms, can be isolated from brains of different Alzheimer's patient. Interestingly, some results indicated that these Aβ mixture could be mirrored by the corresponding CSF molecular profile [27]. This observation could allow the recognition of distinct phenotypes through the determination of the pattern of Aβ peptides in CSF. Aim of this project was to study the main molecular mechanisms involved in the generation of different

clinical/neuropathological phenotypes of the disease and possibly provide grounds for a molecular classification of the disease. Following an approach similar to that largely used in the study of prion diseases, a multidisciplinary strategy has been chosen in order to: 1) Identify molecular profiles based on the content of Aβ peptides in brains from AD patients with different clinical and neuropathological phenotypes. 2) Test the hypothesis that some players of neuroinflammation (microglial cells and immunocytokines) are involved in the determination of clinical and pathological phenotypes of AD.

AIM 1. Identification and characterization of molecular profiles of AD based on the content of Aβ peptides in brains from AD patients with different clinical and neuropathological phenotypes: Molecular subtypes of Alzheimer's disease.

2.2 Introduction

The misfolding, the aggregation and the subsequent deposition of Aβ peptides in brain parenchyma and vessel walls are considered the main events in the pathogenesis of AD [266, 103]. Aβ fragments of several different lengths derived from the cleavage of the amyloid precursor protein (APP) by β- and γ-secretases, and from the consequent digestion by endogenous proteases. This process ends up with the generation of different N- and C-terminal-truncated Aβ species [266, 101, 50, 205] with the ability to assemble into abnormal aggregates [65, 117, 56]. Many recent studies considered Aβ aggregates as the key players in the pathological cascade of AD [48, 96]. Moreover, it has been proposed that different Aβ assemblies exist, each defined by peculiarities in molecular size, stability and neurotoxic characteristics [128]. However, their specific relevance in AD pathogenesis remains unclear. Additionally, different N- and C-terminal truncated forms of Aβ have been described in brain of AD patients [107, 17]. As for Aβ aggregates, we do not know if different Aβ monomeric isoforms play a role in determining specific molecular AD phenotypes. AD may occur under different phenotypes with distinct clinical presentations, neuroanatomical involvement and neuropathological profiles. Although this phenotypic heterogeneity is more evident in the dominantly inherited forms, it is also present in sporadic cases. The molecular bases of these phenotypic variations are far to be completely understood [49, 146, 177]. Previous studies reported that Aβ deposits have different morphology and biochemical composition among individuals with AD as well as among AD transgenic mouse models [150, 196, 157, 180, 170, 260]. The existence of different Aβ "morphotypes" is also supported by the presence of distinct structural variants of Aβ fibrils in brain of AD patients [186, 156]. It is suggested that diffusion of Aβ aggregates from region to region may be responsible for disease process and neurodegeneration propagation, with mechanisms similar to

the spreading of aberrant forms of the prion protein (PrPRes) in transmissible spongiform encephalopathies [130]. This hypothesis relies on the experimental evidence that Aβ amyloidosis can be induced in animal models through the inoculation of brain extracts containing Aβ assemblies [169, 19], and distinct species of aggregated Aβ can reproduce the neuropathological profile of the donor in a given transgenic mouse line [106]. The objectives of this study were: (i) to examine whether a molecular heterogeneity based on the existence of distinct profiles of Aβ aggregates occurs in AD; (ii) to characterize the molecular features delineating different Aβ assemblies and investigate whether the differences in molecular profiles alter the physicochemical properties of Aβ aggregates, that can be involved in the generation of different AD subtypes. We found that either in the sporadic and genetically determined forms of AD, amyloid aggregates have differences in their biochemical composition, determined by their content in different Aβ species. These biochemical variances modulate several properties of Aβ assemblies, which include aggregation kinetics, resistance to proteases action, in vitro seeding activity, and capability to trigger amyloidosis in animal models. These evidences sustain the hypothesis that the variability of AD phenotypes may come from different Aβ aggregation modalities. Hence, an exhaustive analysis of Aβ aggregation and seeding properties may give rise to a new classification of AD, based on the identification of subtypes of Aβ's distinct macromolecular aggregates.

2.3 Materials and Methods

2.3.1 Selection and neuropathological characterization of AD cases

Characterization of AD cases (n = 24) was mainly determined by their neuropathological changes and taking into consideration burden, morphology and localization of amyloid plaques, relative percentage of parenchymal and vascular deposits, immunoreactivity for a panel of antibodies able to recognize epitopes of different Aβ peptides. All samples were screened for the ApoE genotype. Genetically determined AD cases were classified as fAD1 to fAD4. Sporadic cases were classified as sAD1 up to sAD20.

2.3.2 Amyloid extraction

Completed protocol. Amyloid was isolated from frozen brain tissue of five AD patients as previously reported for PrP amyloid [251] and applied to β-amyloid. Specifically, 8 g of frontal cortex were serially homogenized in 9 volumes of buffer A

(10 mM Tris-HCl, Sigma-Aldrich, St. Louis, MO pH 7.5, 150 mM NaCl, Sigma-Aldrich, St. Louis, MO, 1% Triton X-100, Amresco, Solon, OH, added with Complete Protease Inhibitors cocktail, Roche, Mannheim, Germany), buffer B (10 mM Tris-HCl pH 7.5, 0.6 M KI, Sigma-Aldrich, St. Louis, MO, 0.5% Triton X-100, Complete Protease Inhibitors cocktail) and buffer C (10 mM Tris-HCl pH 7.5, 1.5 M KCl, Sigma-Aldrich, St. Louis, MO, 0.5% Triton X-100, Complete Protease Inhibitors cocktail). At the end of each step, the homogenate was centrifuged at 10,000 xg for 40' at 4 °C, the pellet washed four times in buffer D (50 mM Tris-HCl, 150 mM NaCl, pH 7.5), centrifuged at 55,000 xg for 40 minutes at 4 °C, and treated with Collagenase Type1 at 37 °C for 18 hours. After centrifugation at 70,000 xg for 1 hour at 4 °C, the pellet was washed three times in 50 mM Tris-HCl pH 7.5, loaded on a discontinuous sucrose gradient (1.0, 1.2, 1.4, 1.7, 2.0 M sucrose, Sigma-Aldrich, St. Louis, MO, in 10 mM Tris-HCl pH 7.5) and centrifuged at 130,000 xg for 2 hours at 20 °C. Each interface was collected, washed three times in buffer D and centrifuged at 55,000 xg for 30 minutes at 4 °C. Amyloid was then extracted with 80% formic acid, Sigma-Aldrich, St. Louis, MO, dried and finally re-suspended in H2O for further investigations. Simplified protocol. A simplified amyloid extraction protocol was also used to the same five AD samples. In this case, 300 mg of frontal cortex were homogenized in 9 volumes of 10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100 added with Complete Protease Inhibitors cocktail using a the Dounce homogenizer, sonicated for 2 minutes using a Ultrasonic homogenizer Sonopuls-series HD2070 and centrifuged at 3,000 xg for 5 minutes at 4 ° C. The supernatant was loaded on a discontinuous sucrose gradient (1.0, 1.4, 1.8 M sucrose in 10 mM Tris-HCl pH 7.5) and centrifuged at 130,000 xg for 2 hours at 20 ° C. Every interface was collected, washed three times in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 and centrifuged at 55,000 x g for 30 minutes at 4 ° C. The pellet was treated with 80% formic acid, dried and re-suspended in H2O for further investigations. No significant differences came up between the full and the simplified sucrose-gradient fractionation protocols (data not shown). Thus, to extract amyloid from all the other AD cases and controls the simplified procedure was used.

2.3.3 Immunoproteomic analyses

This analysis for Aβ isoforms detection was made as previously reported [3], with few adjustments. 3 μl of a 0.125 mg/ml monoclonal antibody mixture (6E10 and 4G8, Covance, Dedham, MA) was incubated for 3 h at room temperature (RT) in a humidity chamber to permit covalent binding to the PS20 ProteinChip Array (Bio-RAD Laboratories Inc., Hercules, CA). Unreacted spots were blocked for 1 h at RT with 0.4 M Tris-HCl, pH 8.0, in a humidity chamber. Each site was washed three times using PBS with 0.5% (v/v) Triton X-100 then twice using only PBS. Spots were covered with 5 μl of sample and incubated at 4 ° C overnight (ON)

in a humidity chamber prior to be washed three times with PBS with 0.1% (v/v) Triton X-100, twice with PBS alone and finally with deionized H2O. 1.2 μl of α-cyano-4-hydroxy cinnamic acid (Bio-RAD Laboratories, Inc., Hercules, CA) was added to each site and mass characterization was done using the ProteinChip SELDI System, Enterprise Edition (Bio-RAD Laboratories, Inc., Hercules, CA). Different amyloid profiles characteristic of each AD subgroup were recognized considering the relative percentage of the different Aβ isoforms detected by surface-enhanced laser desorption/ionization Time-of-flight mass spectrometers SELDI-TOF MS.

2.3.4 Brain homogenates

Brain homogenates were obtained as previously described [127]. Shortly, 200 mg of frontal cortex were homogenized in 5 volumes of 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, added with Complete Protease Inhibitors cocktail and Phosphatase Inhibitors Cocktail 2 (Sigma) utilizing the Dounce homogenizer and ultracentrifuged at 100,000 xg for 1 hour at 4 $^\circ$ C. The supernatant was collected, aliquoted and stored at -80 $^\circ$ C as the S1fraction. The pellet was re-homogenized in 1% Chaps, 1% Deoxycholate, 0.2% SDS, 140 mM NaCl, 10 mM Tris-HCl, pH 7.5, added with Protease and Phosphatase Inhibitors and ultracentrifuged at 30,000 xg for 30 minutes at 4 $^\circ$ C. The supernatant was aliquoted and stored at -80 $^\circ$ C as the S2 fraction; the pellet was homogenized in 2% SDS, 20 mM Tris-HCl, pH 7.5, 140 mM NaCl and ultracentrifuged at 30,000 xg for 30 minutes at 4 $^\circ$ C. The supernatant was collected as the S3 fraction and stored at -80 $^\circ$ C; the pellet was extracted in 4% SDS, 8 M Urea (P3 fraction). The total proteins amount was quantified in every fraction by BCA Protein Assay kit (Pierce). Immunodepletion was performed by using Protein G Mag Sepharose beeds (GE Healthcare) and a solution of 4G8 and 6E10 antibodies.

2.3.5 Thioflavin T aggregation assay

For the Thioflavin T aggregation assay 5 μL of brain homogenates' soluble fractions (S1) were diluted in 100 mM Tris-HCl pH 7.5, 5 μM ThT, and dispensed in triplicate into wells of a black, clear bottom, 96-well microplate (Nunc). The plate was incubated at RT into a BMG Fluostar Optima Microplate Reader (BMG Labtech). Every 59 minutes the plate was shacked for 60 seconds and the fluorescence was taken. To compare aggregation kinetics among Aβ isolated from different AD brains, we took into account the slope of the curve derived by fluorescence values at separate time points. A prior step in the ThT assays was made to assess the effects of the first quantity of soluble Aβ in AD brain extracts on the shape of aggregation curves. To do this, 5 or 10 μL of brain homogenates' soluble fractions (S1) of only certain human brain samples were analysed with this assay revealing comparable profiles.

2.3.6 Real-Time Quaking-Induced Conversion (RT-QuIC) assay

For these study, an already described protocol [223] - not yet fully validated – was utilised. Few modifications were made to be in accordance with the analysis of brain samples. 5 μL of brain homogenates' S1 fractions were used as seeds and diluted in 100 mM Tris-HCl pH 7.5, 5 μM ThT, 4 μM synthetic Aβ1-42WT or Aβ1-42A2V. The reactions were dispensed in quadruplicate into wells of a black, clear bottom, 96-well microplate (Nunc). The plate was incubated at 37 °C into a BMG Fluostar Optima Microplate Reader (BMG Labtech), and shaken every minute. Fluorescence was taken every 15 minutes. The same protocol was used to assess the seeding capability of brain homogenates on Aβ1-40WT, with the exception for Aβ1-40 concentration (10 μM) and incubation temperature (30 °C).

2.3.7 Sensitivity to PK digestion

For proteinase K (PK) digestion, 5 μg (or 1 μg for patients with A673V and A713T APP mutations) of total proteins from P3 fractions were digested with increasing levels (between 0 and 100 μg/ml) of PK for 1 hour at 37 °C. PK digestion was blocked with the addition of Bolt LDS Sample Buffer (Invitrogen) and Bolt Sample Reducing Agent (Invitrogen) and incubating 10 minutes at 70 °C; the samples were analysed by Western blot.

2.3.8 Western blot

Western Blot analysis was performed loading samples on Bolt 4–12% Bis-Tris poliacrylamide gels (Invitrogen), and then transferred to PVDF and immunoblotted with 4G8 antibody (Signet) (1:1000). Membranes were incubated with biotin-goat anti-mouse (Invitrogen) and Streptavidin-horseradish peroxidase conjugate (GE Healthcare) and revealed through ECL Prime (GE Healthcare). The signal intensity was estimated by densitometry through the software Quantity One (BioRad).

2.3.9 Transmission studies

Whole brain homogenates from AD cases belonging to the different molecular subgroups identified by biochemical studies (AP1-AP3 and the sAD1 case) and two controls (one age-matched nondemented sample and one AD brain homogenate deprived of Aβ by immunodepletion with anti-Aβ antibodies) were prepared as described. Pieces of frontal cortex were homogenized in 9 volumes of sterile 1×

PBS using the Dounce homogenizer, sonicated for 15 seconds using the Ultrasonic Sonopuls-series HD2070 homogenizer and centrifuged at 3,000 xg for 5 minutes at 4 °C. The supernatants were not discarded at this time but aliquoted and stored at -80 °C until injection in APP23 mice (carrying the double Swedish human APP mutation), knock-out for endogenous App (moApp0/0/APP23+/-), chosen in order to bypass the interference of murine App in the spreading of the pathology. See Supplementary Information for details.

2.3.10 Statistical analysis

Student t-test was chosen to compare amyloid burden in immunohistochemical studies on human brains and on brains from mice inoculated with human cerebral homogenates. The densitometric data obtained from the quantification of Western Blot for the study of PK resistance were compared by two-ways ANOVA. Kruskal-Wallis followed by Dunn's multiple comparison test was used to compare Aβ40 and Aβ42 levels measured by ELISA in the insoluble fractions of injected mice. Two tailed P value less than 0.05 was considered statistically significant. All calculations were performed using GraphPad Prism 5.

2.4 Results

2.4.1 Neuropathological characterization of beta-amyloidosisamong AD cases

A neuropathological study in a cohort of 20 sporadic AD patients (indicated as sAD1-sAD20 numbered in Table 2.1) and 4 genetically determined AD patients with mutation in APP, PS1 or PS2 genes - fAD-APPA673V, fAD-APPA713T, fAD-PS1P117A and fAD-PS2A85V – (indicated as fAD1–4 in Table 2.1), revealed the typical changes of AD such as the parenchymal (amyloid plaques) and vascular (congophilic amyloid angiopathy, CAA) amyloid deposits, neurofibrillary tangles, neuropil threads and dystrophic neurites with hyperphosphorylated tau, together with neuronal loss, astrogliosis and activation of the microglia within the cerebral cortex. However, a more exhaustive examination of the amyloid-β pathology showed the existence of differences in density, shape and size as well as in the relative severity of parenchymal versus vascular deposition of amyloid in the brain (Figure 2.1). Particularly, the A673V mutation (fAD1) (a,f,k panels), whose full neuropathological investigation was previously described [78], revealed huge amyloid deposits in the parenchyma as well as in the vessels, which were immunoreactive for antibodies that recognize epitopes throughout the Aβ sequence. Several small vessels within the parenchyma and leptomeninges showed a decrease of the walls' thickness because of the accumulation of amyloid and 'drusige Entartung'. Pathological features of APPA713T (fAD2) (b,g,l panels) were CAA and low-density parenchymal Aβ amyloid aggregates in the neuropil. Severe amyloid deposition was present in leptomeningeal and small parenchymal vessels in the cerebral hemisphere. Affected vessels were disrupted with amyloid assuming a radial appearance, thickening and double barreling of the wall, loss of smooth muscle cells and narrowing of the lumina. Neuropathological assessment of the PS1P117A case (fAD3) (c,h,m panels) was characterized by the profuse and widespread plaques in all cortical layers with higher density in the subpial region. The two sporadic cases displayed completely dissimilar amyloid profiles, one (sAD1) (d,i,n panels) with preponderance of vascular amyloid deposits and capillary Aβ deposition going from the vessel walls into the surrounding neuropil ('drusige Entartung'), and mature plaques few and far between the cerebral cortex, the other (sAD6) (e,j,o panels) with small and diffuse plaques spread over all cortical layers, with focal and mild CAA. This findings suggested that, just taking into consideration the morphology and the distribution of Aβ aggregates, the neuropathology of AD is variable not only among familiar cases but also among sporadic ones.

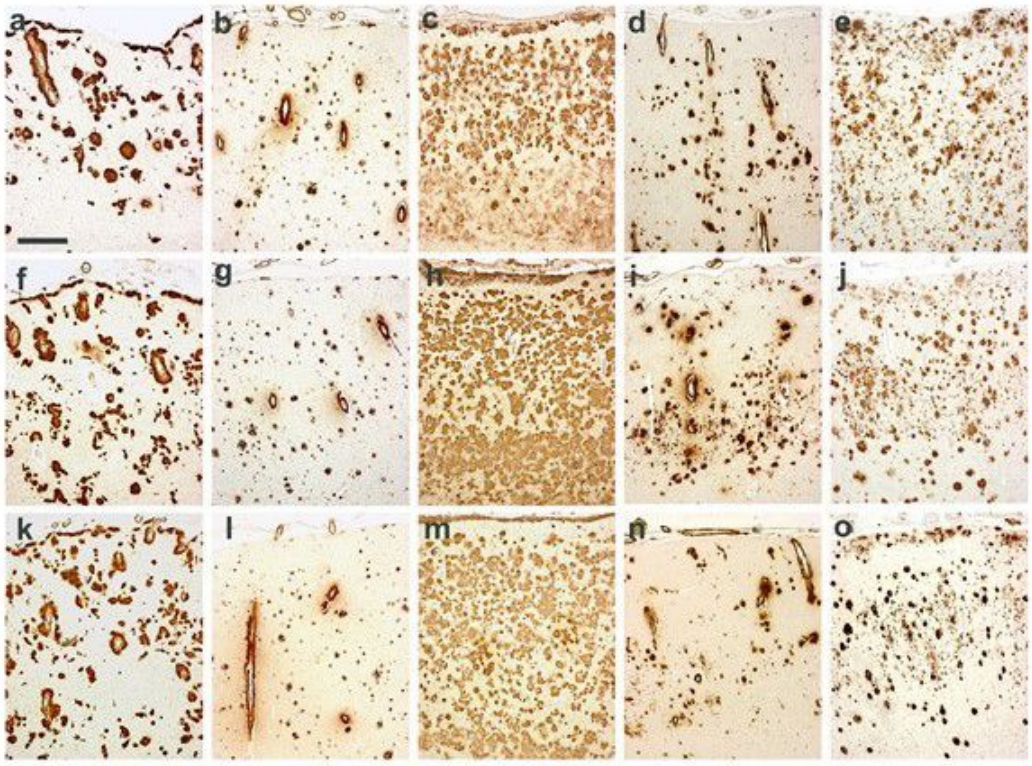

Fig. 2.1: Neuropathological differences of amyloid deposition in AD cases. (a,f,k) APPA673V (fAD1 in Table S1); (b,g,l) APPA713V (fAD2 in Table 2.1); (c,h,m) PS1P117A (fAD3 in Table 2.1); (d,i,n) sAD carrying the ApoE $\epsilon 4/\epsilon 4$ genotype (sAD1 in Table 2.1); (e,j,o) sAD $\epsilon 3/\epsilon 3$ (sAD6 in Table 2.1). Scale bar = 400 μm. (a,b,c,d,e) frontal cortex; (f,g,h,i,j) temporal cortex; (k,l,m,n,o) occipital cortex. Immunohistochemical study perfomed using the 4G8 antibody directed to Aβ peptide.

Case	Age at onset	Age at death	ApoE/APP/PS1/PS2 genotype	**Braak NFT stage**	**CAA**	**Plaque density score**	**Dispersion Index frontal cortex**	**Aβ38**	**Aβ40**	**Aβ42**
fAD1	36	46	$\epsilon 3/\epsilon 3$, APP A673V	VI	3	3	92,05	++	++	++
fAD2	52	57	$\epsilon 3/\epsilon 3$, APP A713T	VI	4	2	123,3	++	++	++
fAD3	36	43	$\epsilon 2/\epsilon 3$, PS1 P117A	VI	3	3	214,5	-	+	+++
fAD4	60	82	$\epsilon 3/\epsilon 3$, PS2 A85V	VI	0	3	239,3	-	-	++
sAD1	79	82	$\epsilon 4/\epsilon 4$	V	4	3	96,7	+	++	+
sAD2	65	68	$\epsilon 3/\epsilon 3$	V-VI	1-2	2	43,55	+/-	++	++
sAD3	n.a.	59	$\epsilon 3/\epsilon 3$	VI	1-2	3	143,76	-	++	++
sAD4	79	81	$\epsilon 3/\epsilon 4$	III-IV	2-3	3	108,53	-	-	++
sAD5	77	83	$\epsilon 3/\epsilon 4$	VI	3	1	153,84	-	+/-	++
sAD6	60	75	$\epsilon 3/\epsilon 3$	VI	1	3	65,21	+/-	++	++
sAD7	62	72	$\epsilon 3/\epsilon 3$	VI	3	3	240,3	-	+	++
sAD8	62	68	$\epsilon 2/\epsilon 4$	III-IV	2	2	78,38	+	++	++
sAD9	83	86	$\epsilon 3/\epsilon 4$	IV	3	3	218	-	+/-	++
sAD10	85	90	$\epsilon 3/\epsilon 3$	III	1	3	365,57	-	+/-	++
sAD11	53	58	$\epsilon 2/\epsilon 3$	V-VI	2	2	163,5	-	+	++
sAD12	50	58	$\epsilon 3/\epsilon 3$	VI	3	3	174,4	-	+/-	++
sAD13	58	66	$\epsilon 4/\epsilon 4$	VI	3	3	125,3	+/-	+	++
sAD14	63	69	$\epsilon 2/\epsilon 4$	VI	3	3	120	-	+/-	++
sAD15	54	62	$\epsilon 2/\epsilon 3$	VI	2	3	165,1	-	+/-	++
sAD16	43	47	$\epsilon 4/\epsilon 4$	V-VI	2-3	3	146,3	-	+	++
sAD17	51	59	$\epsilon 3/\epsilon 4$	V	2	3	133	-	+	++
sAD18	47	61	$\epsilon 3/\epsilon 3$	VI	3	3	157,3	-	+	++
sAD19	69	72	$\epsilon 3/\epsilon 4$	V-VI	2	3	277	-	++	++
sAD20	82	86	$\epsilon 3/\epsilon 4$	IV	3	2	118.9	+/-	+/-	++

Tab. 2.1: Neuropathological profiles of AD cases.

2.4.2 Identification of AD molecular subgroups based on the Aβ content of amyloid assemblies

Based on the working hypothesis that the variability in Aβ pathology might be due to differences in the molecular composition of amyloid, we isolated parenchymal amyloid from the brains of the sAD1–20 and the fAD1–4 patients and examined each Aβ content by immunoproteomic analysis based on the use of SELDI-TOF MS. The investigation showed how different Aβ isoforms, including N- and C-terminally truncated Aβ species, contribute to amyloid composition and that the relative quantity of each peptide can vary among AD brains. These data allow us to identify two major AD subgroups, each identified by distinctive Aβ profiles (Table 2.2 and Figure 2.2), indicated as Amyloid Profile 1 (AP1) and Amyloid Profile 2 (AP2). AP1 was found in 14 sAD patients and in the individuals with mutations in PS1 and PS2, while AP2 was found in five sAD cases. AP1 was characterized by an elevated relative proportion of AβX-42 peptides, especially Aβ1-42, Aβ4-42, Aβ11-42, the pyroglutamate-modified Aβ3pE-42 and the Aβ11pE-42, while AP2 was marked by the presence of abundant AβX-40 peptides, AβX-42 peptides, and by a lower amount of N- and C-terminal truncated forms, such as Aβ2-39. The two cases with the APP mutations displayed a distinct amyloid profile, designated as AP3, mainly represented by Aβ1-40, Aβ1-38 and Aβ1-37 peptides. Eventually, one individual with sAD (sAD1), with a very severe CAA (panels d,i,n in Figure 2.1), showed a peculiar profile with a preponderance of Aβ1-40, Aβ3pE-40 and Aβ1-36 isoforms. Based on these data, Table 2 shows the molecular stratification of our AD cases into three distinct subgroups (AP1-AP3). sAD1, whose amyloid profile cannot be comprised in the other groups, is also showed. Neuropathological studies provided evidence of heterogeneity of patterns of Aβ deposition among both familial and sporadic AD patients (Table 2.2). Anyway, when we looked for association between the molecular and the neuropathological profiles within each AD subgroup, we did not find a clear-cut correlation between the two variables. Likely, the pathologic changes found in AD brains may be the result of a combination of molecular determinates that include not only Aβ species but also additional molecules, such as the tau protein and other unknown factors.

Profile	Case	Main Aβ peptides	Apo E genotype
AP1	fAD3($PS1_{P117A}$), fAD4($PS2_{A85V}$), sAD2, sAD3, sAD4, sAD5, sAD6, sAD7, sAD8, sAD10, sAD12, sAD14, sAD15, sAD18, sAD19, sAD20	Aβ1-42, Aβ4-42, Aβ3pE-42, Aβ11pE-42	ε2= 9.4% ε3= 68.7% ε4= 21.9% ε2= 10.0%
AP2	sAD9, sAD,sAD11, sAD13, sAD16, sAD17	Aβ1-40, Aβ1-42	ε3= 30.0% ε4= 60.0%
AP3	fAD1(APP_{A673V}), fAD2(APP_{A713T})	Aβ1-40, Aβ1-38, Aβ1-37	ε3= 100.0%
—	sAD1	Aβ1-40, Aβ3pE-40, Aβ1-36	ε4= 100.0%

Tab. 2.2: Molecular profiles of AD patients based on Aβ peptides content

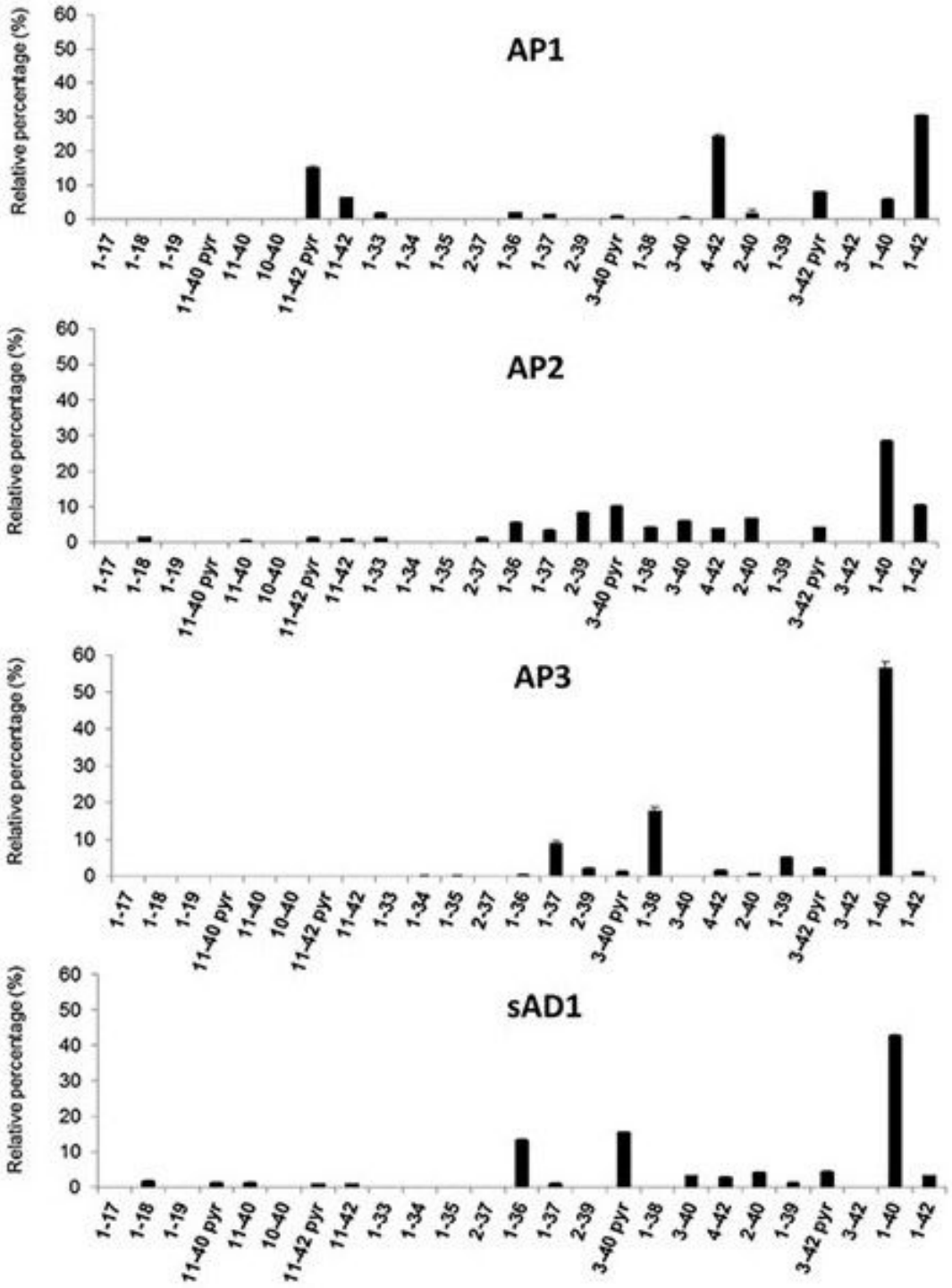

Fig. 2.2: Aβ isoforms profiles. Aβ isoforms were extracted by immunoproteomic assay, using two different Aβ monoclonal antibodies (6E10 and 4G8) on pre-activated chip array, followed by mass spectrometry. Relative percentage of Aβ peptides (with respect to the total Aβ amount) were measured in AD brains; a representative profile for each subgroup is reported (n = 3, mean relative percentages ± SEM (AP1 to AP3 numbering; sAD1 profile is reported outside of the other groups for its peculiarities).

2.4.3 AD molecular subgroups show distinct aggregation pathways

We then examined if the soluble fraction of brain homogenates from AD cases with different Aβ profiles follow distinct aggregatation pattern in vitro using ThT assays. The experiment revealed that the two principal molecular AD subgroups (AP1 and AP2) show different aggregation pathways. Indeed, the AP1 subgroup aggregation kinetics resulted to be faster than the AP2 subgroup. The AP3 subgroup displayed an even slower aggregation than the other subgroups currently with the time-course of the study. On the other side, aggregation kinetics was particularly fast for sAD1 brain sample (Figure 2.3a, b). Brain extracts, upon immunodepletion from Aβ, did not display any aggregation, suggesting that Aβ peptides are the principal species implicated in aggregation in the setting of this assays (data not shown).

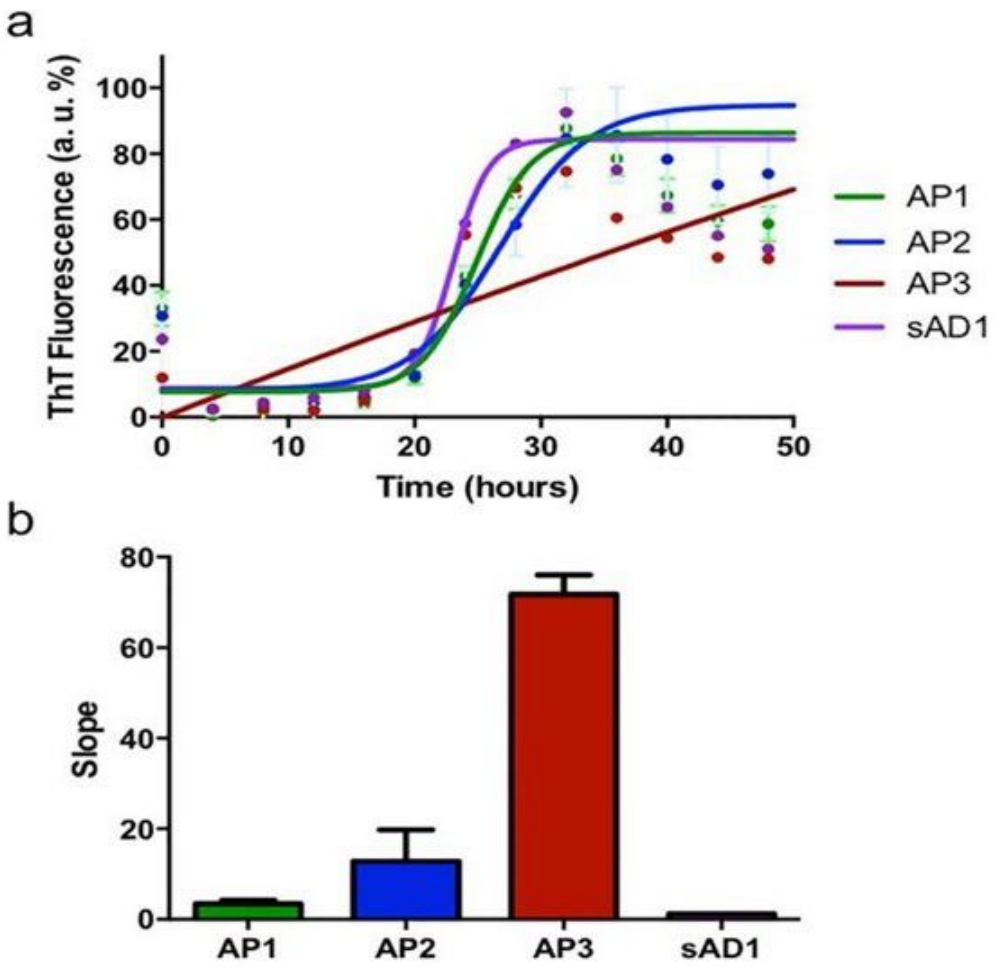

Fig. 2.3: Aggregation pathways of distinct Aβ seeds by ThT assays. Aggregation kinetics of different AD -Aβ profiles were analyzed by ThT assays. Soluble fractions from AD brain homogenates were used for this assay, diluted in 100 mM Tris-HCl pH 7.5, 5 μM ThT, the ThT intensity was normalized to the corresponding maximal ThT fluorescence and the Boltzmann equation was used to fit mesurements (a). Kinetics were normalized and compared by considering the slope curves described by ThT fluorescence emission (b).

2.4.4 AD molecular subgroups display different seeding abilities

RT-QuIC was used to investigate the seeding effects of the AD molecular subtypes on synthetic wild-type Aβ1-42 (Aβ1-42WT) substrate (Figure 2.4a). The analysis showed that brain extracts from patients of the AP1 and AP2 subgroups, and the sAD1 patient have seeding activities. Indeed they are able to shorten the lag phase of aggregation kinetics of synthetic Aβ1-42WT (Figure 2.4a). In particular, AP1 revealed a higher seeding activity compared to AP2 and the sAD1 sample induced the fastest aggregation of Aβ1-42 with a sharp slope in the polymerization step of aggregation kinetics. Differently, brain extracts from AP3 subgroup revealed a week seeding effect on the Aβ1-42WT substrate and followed different aggregation kinetics. Curiously, the aggregation kinetics led by the brain extract from the APPA673V homozygous patient are faster when the mutated Aβ peptide (i.e., Aβ1-42 carrying the A673V mutation)was utilised as substrate in the RT-QuIC analysis, suggesting that the aggregation profile may depend by the affinity between seed and monomer (Figure 2.4b). At the beginning of the experiment, APPA713T reveals high ThT signal. This might be due to a very fast enrolment of the substrate that leads to the premature saturation of ThT signal. The signal decline in the rest of the curve may be due to the instability of the aggregates arose by this brain extract during the analysis. RT-QuIC made on Aβ1-42WT substrate co-incubated with brain extracts from control group (i.e., healthy subjects without neuropathological changes) and from immunodepleted controls (Figure 2.5) did not reveal any aggregation along the time-course of the study. RT-QuIC assay was also performed utilising a synthetic wild-type Aβ1-40 substrate (Figure 2.6). In this case, no significant differences were observed among the brain extracts of the different subgroups, indicating that Aβ1-40 is not a useful in the detection of differences in seeding abilities of the different AD subgroups.

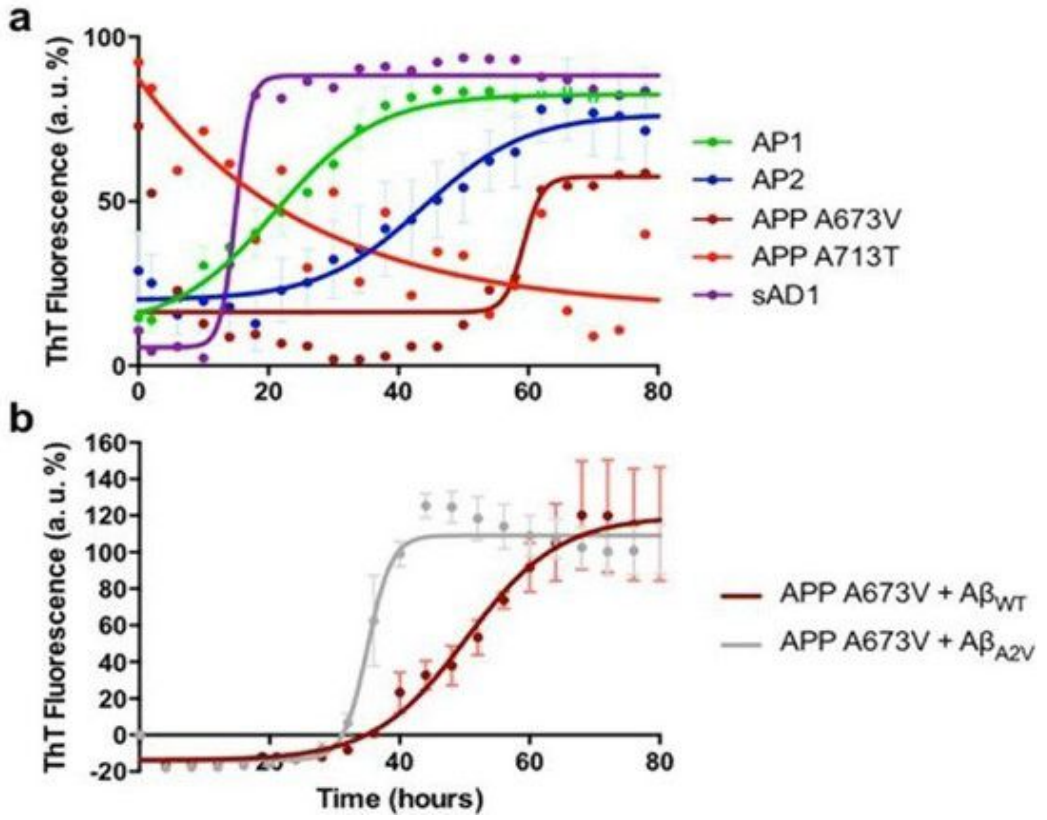

Fig. 2.4: RT-QuIC profiles of AD patients selected from AP subgroups. Soluble fractions from AD brain homogenates were diluted in 100 mM Tris-HCl pH 7.5, 5 μM ThT, 4 μM Aβ1-42WT (panel a) or Aβ1-42A2V (panel and b). ThT fluorescence intensity was normalized to the corresponding maximal ThT fluorescence obeserved and expressed as relative arbitrary units (a. u. %). Aggregation kinetics were compared in (a). Each brain sample was analyzed in quadruplicate. Data are shown as mean ± SEM. Comparison of aggregation kinetics of APPA673V brain extract when co-incubated with Aβ1-42 wild-type or Aβ1-42 (b). Each brain sample was analyzed in triplicate. Data are shown as mean ± SEM.

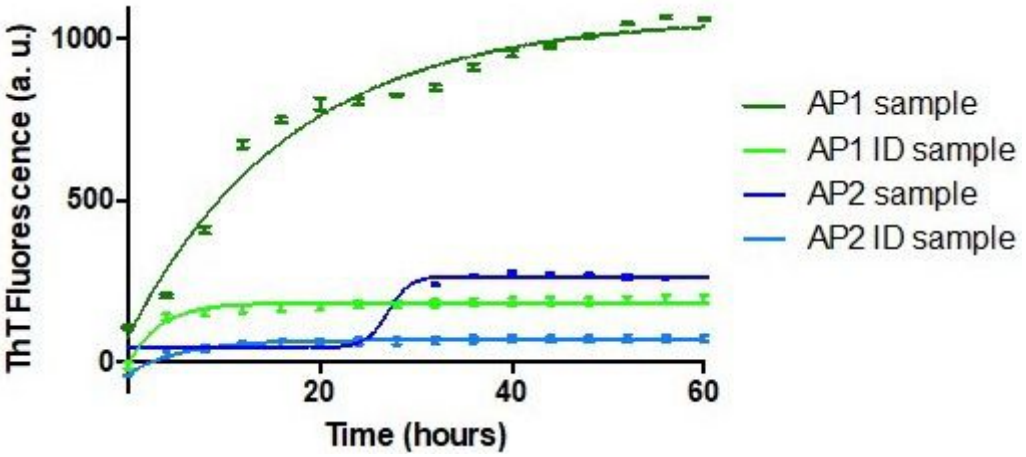

Fig. 2.5: Effects of immunodepletion of Aβ on aggregation kinetics of AD brain homogenates. Soluble fractions from AD brain homogenates were diluted in 100 mM Tris-HCl pH 7.5, 5 μM ThT. ThT fluorescence is expressed as arbitrary units (a. u.).

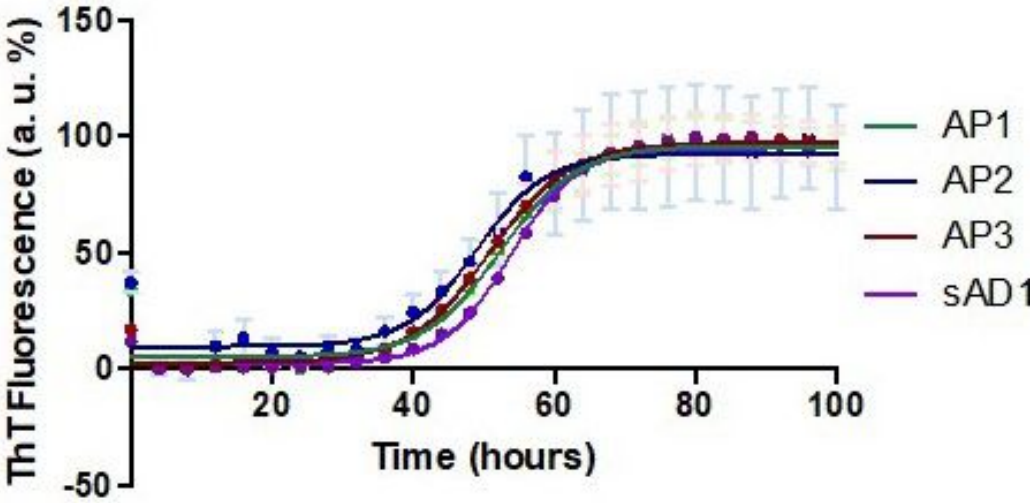

Fig. 2.6: RT-QuIC profiles of human brain extracts from the molecular subgroups of AD with Aβ1-40WT as substrate. Soluble fractions from AD brain homogenates were diluted in 100 mM Tris-HCl pH 7.5, 5 μM ThT, 10μM Aβ1-40. ThT intensity was normalized on the corresponding maximal ThT fluorescence and expressed as relative arbitrary units (a. u. %). Each brain was analyzed in quadruplicate; brains belonging to the same amyloid profile were grouped. Data are shown as mean ± SEM.

2.4.5 AD molecular subgroups show different resistance to PK degradation

The resistance of Aβ aggregates to protease activity was evaluated through the digestion of P3 fraction from AD brain homogenates with increasing amounts of PK. Samples of the AP1 subgroup and the sAD1 case displayed a very high resistance to proteolysis, since high doses of PK (up to 100 μg/ml) did not degrade Aβ aggregates. In contrast, an evident dose-dependent PK degradation of Aβ aggregates was observed in AP2 subgroup samples and in the two APP- mutated cases (Figure 27a, b,). Analogously, for what concern the Aβ dimers digestion (Figure 2.7a, c), the AP1 subgroup and the sAD1 case were quite stable over increasing PK concentrations, AP2 underwent proteolysis in a dose-dependent manner and the dimers from the APP-mutated samples were in part degraded by PK. These data suggest that differences in the Aβ composition of amyloid may affect protease activity on Aβ assemblies, making the Aβ aggregates from distinct molecular subgroups of AD more or less stable.

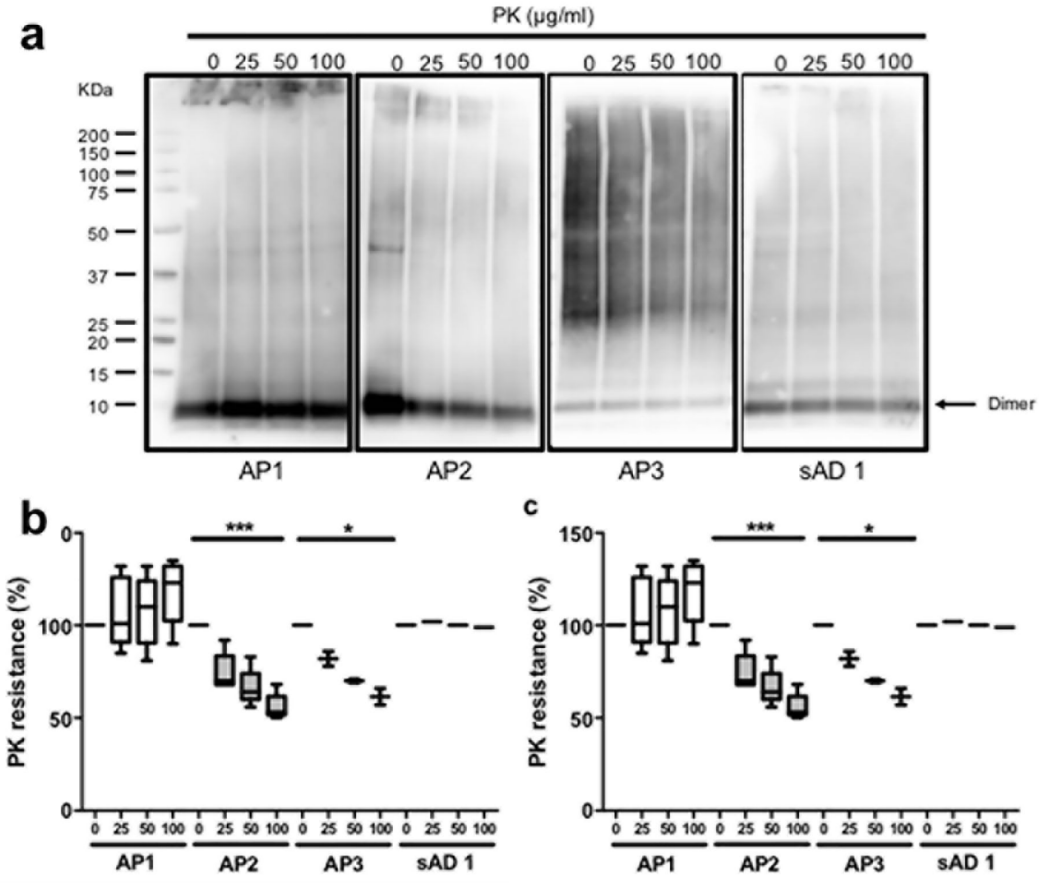

Fig. 2.7: AD subgroups resistance to PK degradation. Insoluble fractions from AD brain homogenates were digested with 0, 25, 50, 100 µg/ml of PK and analyzed by Western blot using 4G8 antibody. The signal intensity of all the Aβ aggregates (a,b) or of Aβ dimers (a,c) was quantified by densitometry; data were analysed by two-ways ANOVA (∗p < 0.05; ∗ ∗ ∗p < 0.001). A representative brain extract has been selected for each amyloid profile.

2.4.6 Intracerebral injection of brain extracts from human AD subgroups in mice results in distinctive pattern of cerebral amyloidosis.

Here we investigated whether the molecular subgroups of AD differently propagate the pathological process in vivo, when injected in animal models [246]. We showed that moApp0/0/APP23+/-, intra-cerebrally inoculated at six months of age with brain homogenates selected among the molecular subtypes previously described, developed brain amyloidoses with different disease profiles as well as different morphology, regional distribution of amyloid aggregates, and preferential parenchymal or vascular Aβ deposition (Figure 2.8 and 2.9), suggesting that the phenotypic heterogeneity of human pathology can be replicated in mice, even if without a close replication of the peculiarity specific of human donor brains. Interestingly, the severity of neuropathological changes induced in mice by the brain extracts from different AD subgroups was dissimilar indicating that Aβ seeds from human brain

homogenates are characterized by physicochemical properties that modify their ability to spread amyloidosis in animal models (Figure 2.8 and 2.9). In fact, AP1 subgroup induced disease propagation more effectively than AP2 subgroup (Figure 2.8). The brain extracts from the two cases with mutations in the APP gene (AP3 subgroup) displayed important discrepancies and thus are separately illustrated in Figure 2.8 and 2.9. Seeds from the sAD1 sample were also highly aggressive (Figure 2.6). AP1 human brain extracts induced an amyloidosis represented by an intense amyloid burden, with small amyloid deposits and spread plaques, heavily associated with diffuse CAA and intraneuronal Aβ immunoreactivity sited in hippocampus and neocortex (a-d panel in Figure 2.9). In this group we noticed abundant Aβ-positive CAA with plaques in the thalamus, a peculiarity already reported by Watts et al. [267] in APP23 mice inoculated with brain extracts from patients carrying the APP 'Artic' mutation. Inoculation of AP2 brain extracts ended up in a weaker and diffuse amyloid deposition, with mild CAA and intracellular immunostaining for Aβ. The thalamus was not involved (e–h panels in Figure 2.9). Amyloidosis induced by APPA713T (fAD2) brain extracts was instead characterized by low amyloid burden, severe CAA and low intraneuronal Aβ immunostaining. In this group as well, the lesion profile revealed a thalamic deposition of Aβ in vessel walls and, to lesser extent, in parenchymal deposits (i–l panels in Figure 2.9). However, the pathologic changes in thalamus were weaker than in mice injected with AP1 brain samples. APPA673V (fAD1) caused only a slight amyloidosis (m–p panels in Figure 2.9). Injection of brain extract from the sAD1 patient was associated with the highest amount of intraneuronal Aβ immunoreactivity, mainly present in hippocampus and motor cortex, and caused the generation of parenchymal amyloid aggregates larger in size, and mild CAA sparing thalamus (q-t panels in Figure 2.9). Intracerebral inoculation of human brain extracts without Aβ seeds did not alter the spontaneous amyloidogenesis of moApp0/0/APP23+/- mice (bottom panel in Figure 2.8 and aa-dd panels in Figure 2.9), reinforcing the theory suggested by previous reports [169, 64, 145] that Aβ seeds are mandatory to speed up amyloidosis in animal models. Injection of human brain samples in non-transgenic control mice did not result in amyloidosis development. Injection of brain extracts from a healthy control did not alter the natural amyloidogenesis of transgenic mice (data not shown). Eventually, second passage inoculations of moApp0/0/APP23+/- mice were performed. The consequent amyloidosis produced in mice maintained the pathologic characteristics induced by previous passage inoculations in each subgroup, showing however a weakening of the severity of neuropathologic changes (data not shown), in accordance with previous studies illustrating the resilience of Aβ seeds [284]. We also assessed Aβ40 and Aβ42 levels in the insoluble fraction of brain extracts from mice injected with human brain homogenates. The results largely confirmed the differences showed by the neuropathological studies with higher Aβ levels in mice injected with AP1 subgroup, APPA713T and sAD1 extracts. The AP2 subgroup

and the APPA673V sample displayed a lower amount of Aβ. The immunodepleted controls revealed Aβ levels like non-injected mice (Figure 2.10).

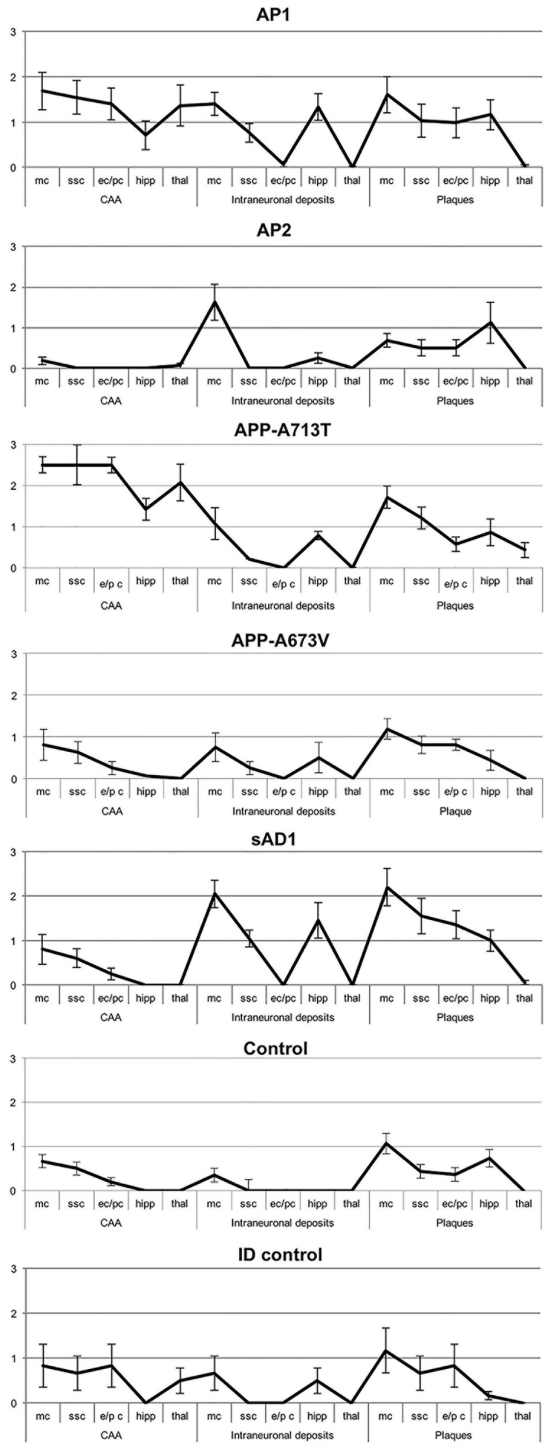

Fig. 2.8: Lesion profiles in mice inoculated with human brain extracts from distinct AD patients. CAA, intraneuronal Aβ immunostaining and amyloid plaques were selected as neuropathological criteria to build up lesion profiles of the disease in mice. Control = untreated age-matched mice. mc = motor cortex; ssc = somato-sensory cortex; ec/pc = enthorinal cortex/piriform cortex; hipp = hippocampus; thal = thalamus. Immunohistochemical study was performed with 4G8 antibody. Quantification of 4G8 immunostaining was calculated by "plaque count" method and expressed in each profile as a mean ± SEM of the values obtained in animal groups (n = 8) injected with human brain extracts of each molecular profile: AP1-AP2 subgroups and APPA673V (fAD1), APPA713T (fAD2) and sAD1 case. Quantification by "plaque count" was carried out using a scale ranging from 0 to 5 by light microscopy. The study was performed using "NIS-elements" software".

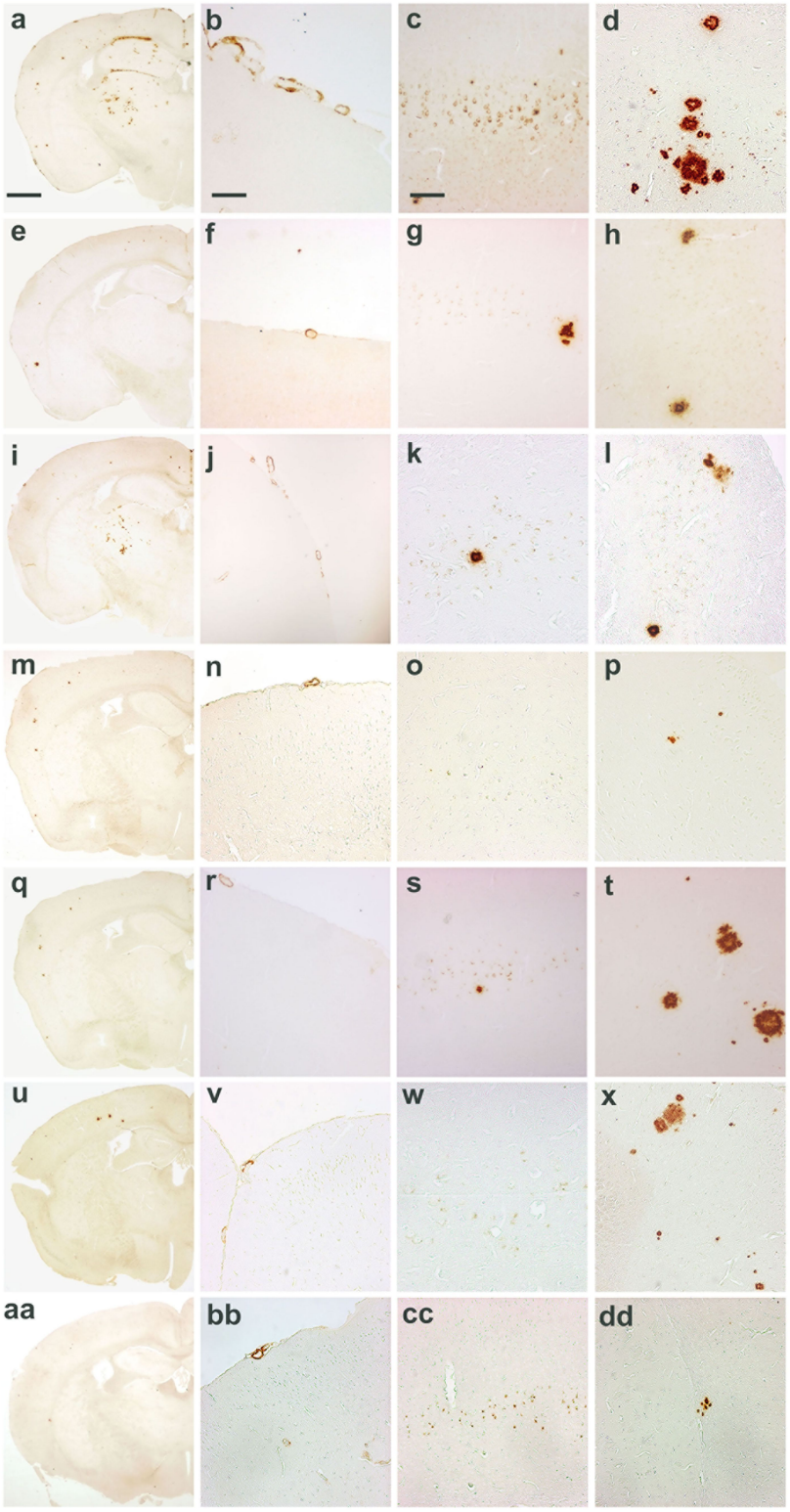

Fig. 2.9: Amyloid burden in mice injected with human brain extracts from distinct AD subgroups. Mice inoculated with AD brain homogenates from AP1 subgroup (a–d), AP2 subgroup (e–h), APPA713T (fAD2) (i–l), APPA673V (fAD1) (m–p), sAD1 case (q–t) and control groups, i.e. age-matched non-injected mice (u–x) and mice injected with Aβ-immunodepleted brain extracts (aa–dd). (a,e,i,m,q,u,aa) Amyloid deposits, scale bar 0,5 mm. (b,f,j,n,r,v,bb) CAA, 300 μm. (c,g,k,o,s,w,cc) Intraneuronal Aβ immunoreactivity, scale bar 120 μm. (d,h,l,p,t,x,dd) Amyloid plaques, scale bar 120 μm. Immunostaining with 4G8 antibody.

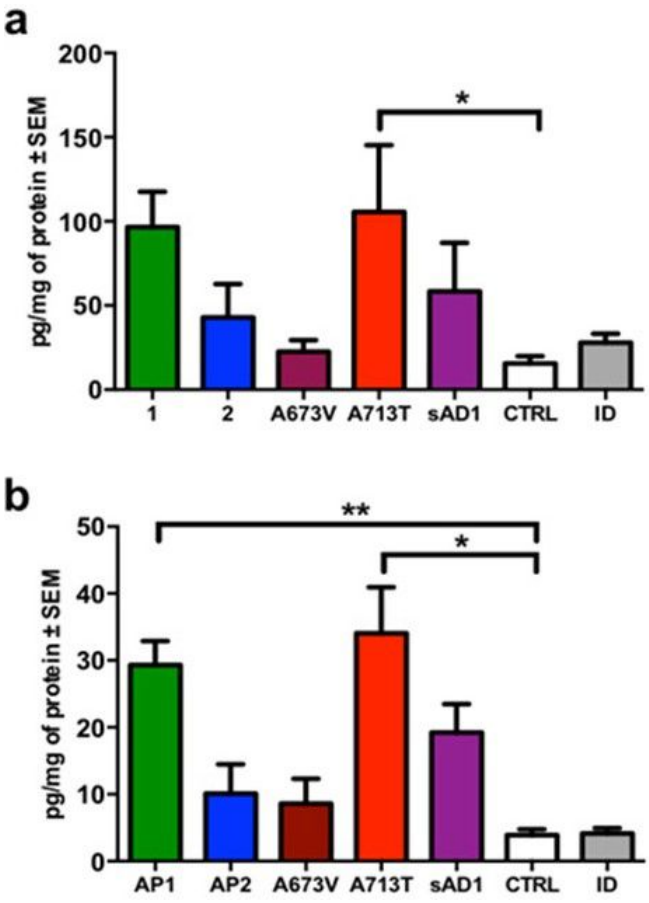

Fig. 2.10: Aβ levels in insoluble fractions from brains of mice injected with human AD brain homogenates. Aβ40 (a) and Aβ42 (b) were measured in duplicate by ELISA and expressed as pg per mg of total proteins. The results were compared by Kruskal-Wallis followed by Dunn's multiple comparison test (∗p<0.05; ∗ ∗p<0.01).

2.5 Discussion

Phenotypic heterogeneity of AD is an intriguing concept whose molecular bases are still largely unknown. Very recently, the Aβ fibril structure variations have been correlated to AD phenotype through solid-state nuclear magnetic resonance assessment on Aβ40 and Aβ42 fibrils obtained by seeded growth from AD brain homogenates. These studies highlighted that there is a qualitative distinction between Aβ assemblies in AD patient's brains and that this discrepancy may identify different AD phenotypes. Our investigation converged the attention on one of the molecular features that may be involved in the origination of differences in the structural properties of Aβ deposits and thus that may be involved in the identification of different AD phenotypes. We found that: (i) different amyloid-β subtypes exist and they are composed by different mixtures of Aβ peptides that take part in the biochemical composition of amyloid; (ii) amyloid-β subtypes might have different aggregation pathways, generating Aβ assemblies with different toxic effects, and aggregation kinetics; (iii) amyloid subtypes also display different seeding activity on monomeric Aβ1-42, depending on the seed-substrate affinity along the polymerization process;

(iv) deposits from distinct subtypes of amyloid-β have dissimilar resistance to proteolysis, giving rise to aggregates that may be stable and toxic in vivo; (v) amyloid subtypes may differently anticipate or accelerate amyloidogenesis in AD animal models. It is reasonable that other environmental elements in the host brain tissue may alter the pathologic changes of human brain-induced amyloidosis in mice, explaining why the mouse pathology is not the simple duplication of the human one. The finding that genetically determined forms of AD (i.e., those associated with mutations in the APP gene) have a peculiar molecular profile compared to sporadic cases is not surprising, however our data boost the impression that molecular heterogeneity also belong to sporadic form [49, 125, 265, 216]. Interestingly, amyloid profile enriched in Aβx-42 peptides (i.e., AP1) showed rapid aggregation kinetics, strong seeding abilities, high resistance to PK digestion and aggressiveness when inoculated in animal models. However, the high concentration of the longest Aβ isoform (i.e., Aβ1-42) in this subgroup cannot fully explicate the aggressiveness of AP1 Aβ seeds. Indeed, sAD1 case, which is characterized by a predominance of Aβx-40 peptides, actually showed the most aggressive molecular phenotype. Moreover, when injected in mice, Aβ seeds from APP mutated patients (AP3 in our molecular grouping) displayed a heterogeneous behaviour, one (APPA713T) being more aggressive, the other (APPA673V) showing only slight aggregation and seeding abilities, inducing weak amyloid deposition in the brain parenchyma. These last findings confirm the results obtained by several studies according to which the heterologous interaction between wt and A673V-mutated Aβ peptides inhibits the Aβ polymerization [54, 55]. These results suggest that the seeding ability, the aggregation property, the resistance to proteolysis and aggressiveness of the neuropathological lesions induced in animal models are caused by the distinct Aβ content that characterize each AD subgroup. Overall these data led to the recognition of 2 main molecular profiles of AD within the cohort of sAD and fAD cases included in our study and sustained a molecular clustering of AD based on the structural and functional properties of the 'amyloids' identified in each subgroup. The overall data obtained by RT-QuIC assays supported the idea that the differences in the aggregation profiles are not simply due to the quantities of Aβ40 or Aβ42 in the specimen used as a substrate (i.e., soluble fraction of brain homogenates), but are reasonably influenced by the nature (i.e., biochemical content) of the initial seed which trigger Aβ polymerization and by the affinity between seed and substrate. Indeed, sAD1, which contains much more Aβ40 than Aβ42 species, largely induce Aβ1-42 polymerization even more than the AP1 subgroup that is much more enriched in Aβ42 isoforms. Furthermore, data on sAD1 case support our hypothesis that some differences in the sAD subgroups may be due to intrinsic properties of the peptides involved into the aggregation process. Based on this hypothesis, different mixtures of Aβ peptides may follow an aggregation pathway distinct from the others, leading to cerebral amyloidosis with specific characteristics. The peculiar features displayed by the sAD1 case also suggest the hypothesis that other types of AD subgroups may exist. Intriguingly,

two very recent papers suggested a structural variability of Aβ aggregates in both sporadic and familial AD patients, supporting our hypothesis on the existence of distinct AD molecular subtypes [214, 38]. Phenotypic differences in AD are probably result of the interaction of a series of elements including genetic risk factors (such as ApoE) and less known environmental factors [144, 162]. In our study, we did not find a correlation between ApoE genotype and amyloid profile. Further studies on larger cohorts of patients and controls are needed to address this point. Use of transmission studies for the identification and characterization of distinct forms of the disease is a still poorly explored approach in the Alzheimer's field36. It has been successfully used for prion diseases to unveil the existence of different prion strains responsible for different prion-related pathologies [267]. Our study arise from previous evidence that injection of human brain extracts into transgenic animals can induce cerebral amyloidosis involving also brain areas far from the injection site [169, 145, 130]. These data suggest that the typical brain abnormalities associated to AD can be induced by a prion-like mechanism based on the propagation of protein misfolding across brain tissue. Moreover, phenotypic variability is a key feature of prion diseases where it has been proved the existence of distinct subtypes of prions [208, 187]. These observations remind to the general concept of prion-like induction and spreading of pathogenic proteins that has been recently expanded to include aggregates of α-synuclein, tau, TDP-43, superoxide dismutase-1, and huntingtin, which characterize several human neurodegenerative disorders such as, Parkinson's/Lewy body disease, fronto-temporal lobar degeneration, amyotrophic lateral sclerosis and Huntington's disease [32, 209]. Interestingly, all these pathologies can present different phenotypes, however the role of the disease-related misfolding proteins in the generation of their phenotypic variability has been poorly investigated [37, 36]. Through by the misfolding PrP adopts different conformations and generate distinct conformers of the protein, which can give raise to different subtypes of prionopathies [209]. Our data hint that AD exists as distinct molecular phenotypes as a result of differences in the mixture of Aβ peptides. Noteworthy, the variances in the amyloid composition offer a sort of fingerprint to identify different molecular AD subgroups. We recently demonstrated that the preferential accumulation of some Aβ fragments brain parenchyma is mirrored by a reduction of the very same fragments in the CSF of AD cases. This offers grounds to the detection of distinct AD subgroups by the analysis of Aβ profile in CSF of AD patients1. The comprehension of molecular mechanisms responsible for the phenotypic diversity in AD is still at the beginning of its history but, considering the emerging evidence that the responsiveness to pharmacological treatments among AD cases could change at least in part to the existence of distinct subgroups of the disease [200, 259], it is becoming an urgent need. According to these findings our study may help to understand the molecular bases of AD heterogeneity and design more appropriate therapies based on recognition of different target phenotypes. The overall data presented above have been recently detailed in a paper published on Scientific Report [71] in March 2018.

3 Neuroinflammation and phenotypic heterogeneity in Alzheimer's disease

AIM 2 Test the hypothesis that some players of neuroinflammation (microglial cells and immunocytokines) are involved in the determination of clinical and pathological phenotypes of AD.

3.1 Introduction

The molecular mechanisms through which Aβ and Tau accumulate in brain tissue and induce neuronal death remain, still nowadays, largely unknown. The "amyloid cascade hypothesis", initially proposed by Hardy et al. in 1992[103], seems to not completely address this question and cannot fully explain the etiology of AD. As showed by neuropathological and imaging studies, amyloid deposition can be also found in cognitively normal elderly subjects[100, 234, 239] Moreover, treatments aimed at reducing the Aβ burden in the brain of both AD patients and animal models did not substantially modify the progression of the disease[112, 222]. These findings suggest that other factors have to be considered, together with Aβ and Tau, in order to understand the molecular machinery that leads to AD development. Supported by findings that polymorphisms in genes encoding immune receptors, such as TREM2[94] and CD33[88], are associated with high risk of developing AD, increasing evidences confer to the neuroinflammation a relevant role in the development of the illness. According to this hypothesis, the understanding of neuroinflammatory mechanisms could add important elements in the comprehension of the disease and at the same time, considering its high heterogeneity, may also be involved in the great variability observed in the AD pathology. Microglial cells may be one of the most important players of neuroinflammation potentially involved in the generation of phenotypic diversity of AD. Morphologically and functionally changeable, these cells constantly survey the surrounding environment protecting the nervous tissue from possible threats. When activated, microglia triggers the immune response releasing several proinflammatory cytokines such as interleukin-1β (IL-1β), IL-12, tumor necrosis factor-α (TNF-α) and numerous endogenous proteolytic enzymes like MMPs, IDE and NEP. The same microglial cells, by secreting anti-inflammatory cytokines

like IL-1ra, IL-4 and IL-10[159], are able to auto-regulate their action, leading the neuroinflammatory process to resolution. In AD, it was demonstrated that microglia are able to bind Aβ starting a massive neuroinflammatory reaction aimed at avoiding the accumulation of Aβ in the brain[188]. However, the continuous overproduction of the Aβ peptide do not allow microglia to stop the inflammatory reaction that, with time, become detrimental. This process probably begins already in the early stages of the disease, when the Aβ starts to accumulate in the brain, and develops through the secretion of pro and anti-inflammatory factors that may modulate the severity of pathology and even contribute to its variability. Taking into account the potential role of the microglia in the development of AD and in its heterogeneity, the objective of this part of the study was to identify molecular/cellular players of neuroinflammation, which might be relevant in the pathogenesis of AD and are potentially involved in the generation of distinct phenotypes of AD. To this end, we carried out a neuropathological analysis of microglia in brain of several AD patients in order to assess differences in morphology, density and distribution that characterize distinct microglial phenotypes. The neuropathological characterization of microglia was complemented by the analysis of pro and anti-inflammatory factors in different biological specimens to investigate the present of specific neuroinflammatory profiles in AD patients.

3.2 Materials and Methods

3.2.1 Samples collection

Brain samples (frozen samples and formalin fixed brain sections) used in this study were collected from 39 AD patients and 36 healthy subjects at the "Fondazione IRCCS istituto neurologico C. Besta" (Milan, Italy) and the Indiana University School of Medicine (Indianapolis, USA). Brain homogenates from frozen samples of 23 AD patients belonged to the AP subgroups previously described by our research team (Di Fede G. et al., 2018), and 6 non-demented control subjects were used for multiplex analysis. Formalin-fixed frontal cortex samples (n = 15) of brains belonged to AD patients chosen among the AP subgroups were used to analyse microglia. Plasma and liquor samples extracted from 20 AD patients (not belonged to AP subgroups) and 15 (CSF) or 20 (plasma) control subjects without inflammatory pathologies were used to analyse the presence of CXCL13 chemokine by ELISA. The control group included the following disorders: headache and migraine (n = 6), hypertension (n = 5), spastic paraplegia (n = 2), ataxia (n = 4), dysphagia (n = 1), dystonia (n = 2), familiar movement disorders (n = 2), non-Langerhans cell histiocytosis (n = 1), hernia (n = 3), aneurysm (n = 2), myopathy (n = 1), stroke (n = 1), Trigeminal neuralgia (n = 1) white matter lesions (n = 1), superficial siderosis (n = 1) and

neuropatic pain (n = 2). Neuropathological characterization Fiftheen out of the 24 AD patients selected among the molecular subgroups previously identified[71] were enrolled in this analysis. Brains samples of patients were obtained at autopsy. Part of the frontal cortex was fixed in 4% formalin, dehydrated in graded ethanol, cleared in xylene, embedded in paraffin, cut in 4-μm-thick sections using a Semi-automatic precision CUT 5062 microtome (SLEE Medical GmbH, Germany) and incubated at 37°C overnight. Sections were then incubated at 56°C for 12 min and gradually hydrated in increasing ethanol percentage solutions. Characterization of AD cases as previously reported by Di Fede G. et., al 2018. Routine examination was carried out on sections stained with hematoxylin–eosin (H&E) and cresyl violet for Nissl substance.

3.2.2 Immunohistochemistry

Immunohistochemistry was carried out using the anti-IBA1 antibody that recognize both the activated and the resting phenotype of microglia. Sections were subjected to antigen retrieval in a Tris-EDTA solution at pH 9 by means the Pt-link instrument (Dako, Agilent) to optimize the epitope unmasking. Slices were incubated 1 hour at room temperature (RT) in a rabbit polyclonal antibody (Wako; 1:400). The immunoreactions were visualized by the EnVision Plus/Horseradish Peroxidase system for rabbit immunoglobulins (Dako, Agilent) using 3-3'-diaminobenzidine as chromogen. All sections were counterstained with Hematoxylin before being cover-slipped. Omission of the primary antibody resulted in the absence of immunostaining.

3.2.3 Image analysis

Digital images of slides were acquired by means of Aperio Scanscope XT (Leica Biosystems, Nussloch, Germany) with a resolution of 40X. Images were then analysed with the ImageScope software: ten regions of interest (ROI) designed as approximate 1 million μm^2 squares were randomly designed in the grey matter of our samples (Figure 3.1a). The Positive Pixel Count V9 algorithm was selected for the analysis of the number and intensity of strong positive pixel. Input parameters are shown in figure 1b. The morphometric analysis was carried out in DAB-stained microglial cells labelled with IBA-1 antibody by ImageJ software.

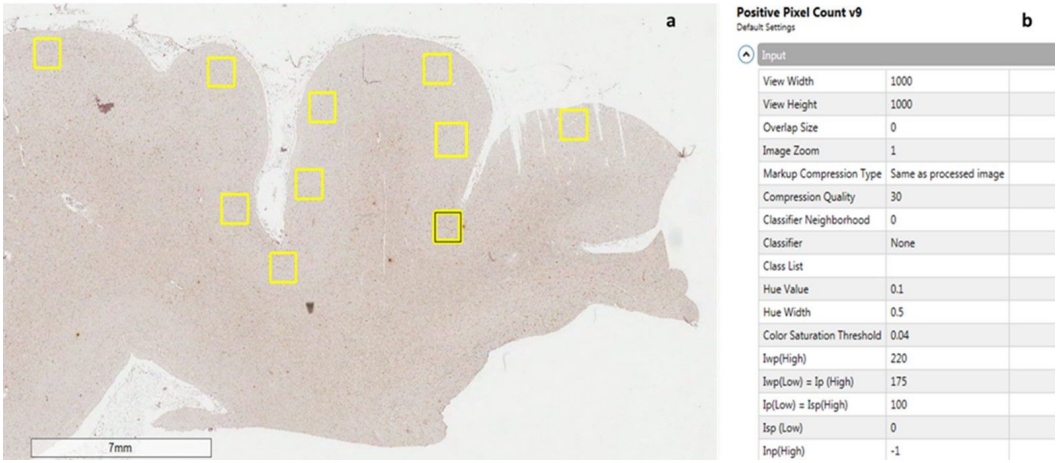

Fig. 3.1: Image analysis procedure. Picture (a) show a representative IHC with anti-IBA1 antibody to stain microglia in an AD sample. Image was acquired by digital scanner and analysed with image scope software (Leica Biosystems, Nussloch, Germany). Yellow squares indicate the ROI considered for the analyses. All ROIs were designed with the same dimension and randomly distribute in the grey matter of the frontal cortex. Table in (b) indicate the input parameters chosen to better underline and therefore quantify the microglial content within the ROIs.

3.2.4 Multiplex arrays

Luminex Human Magnetic Assay (25-Plex) LXSAHM-25 (Bio-Techne/R&D system, Minneapolis, USA) was used to check the presence of 25 factors (Table 3.1), including cytokines, chemokines and MMPs, released during the neuroinflammatory process in AD by microglia. Brain samples were suspended in 9 volume of PBS 1x and manually homogenized by potter in ice. Brain homogenates were centrifuged for 15' at 1500 x g, 4°C and supernatant collected and stored at -20°C. 50 μL aliquots of standard or samples of were added in duplicate to the 96-well plate provide by LXSAHM-25 assay kit. All reagents were prepared according to manufacturer instructions without modifications as well as the assay procedure. The Spike/Recovery test and the Linearity test were performed to assess the matrix interference and to find the right dilution for our samples respectively in order to validate brain homogenates (kind of sample not reported in the datasheet) as proper substrate to analyse. The results indicated no matrix interference and samples were loaded undiluted. For those analytes below the Minimum Detection Dose (MDD) and those out of range (<OOR), the MDD were used for individual samples and cytokines. For those analytes Over of Range (>OOR), the upper limit values of the quantitative range of the assay (the higher calibrator value) were used as well for individual samples and cytokines.

Type	Analyte	Sensitivity (MDD)pg/mL	Standard Curve Range (pg/mL)	Dilution	Sample (μL)
Cytokine	INF-γ	0.4	58.5-14,209	1:1	50
	IL-1α	0.9	5.2-1,270		
	IL-1 ra	18.0	28-6,793		
	IL-1β	0.8	19.5-4,744		
	IL-2	1.8	29.6-7,200		
	IL-4	9.3	14.6-3,550		
	IL-6	1.7	4.8-1,154		
	IL-10	1.6	4.8-1,162		
	IL-12 p70	20.2	137.2-33,340		
	IL-13	32.4	455-110,520		
	IL-18	1.93	54.6-13,260		
	TNF-α	1.2	9.7-2,359		
Chemokine	CCL2/MCP1	9.9	333-8,017	1:1	50
	CCL5/RANTES	1.8	22.6-5,488		
	CCL17/TARC	6.47	85.6-20,790		
	CX3CL1/Fractalkine	64.8	1,114-270,610		
	CXCL9/MIG	18.4	586-142,400		
	CXCL10/IP-10	1.18	2.8-690		
	CXCL13/BLC/BCA-1	11.5	12.6-3,060		
Matrix Metalloproteinase (MMP)	MMP-1,	2.7	49.7-12,085	1:1	50
	MMP-7	23.2	350.1-85,082		
	MMP-8,	34.2	245.1-59,562		
	MMP-9	13.6	134.1-32,596		
Innate Immunity factors	Lipocalin-2/NGAL,	29.2	140-33,920	1:1	50
	CD14	39.6	202-49,180		

Tab. 3.1: Samples and reagent preparation. The minimum detection dose and the standard curve range are reported for all the analytes tested. Interferon (INF), interleukin (IL), Tumor necrosis factor (TNF), chemokine (C-C motif) ligand (CCL), Chemokine (C-X-C motif) ligand 9 (CXCL), Matrix metalloproteinase (MMP), Cluster of differentiation (CD).

3.2.5 ELISA

CXCL13 levels in 20 cerebrospinal fluid (CSF) and Plasma (Table 3.2) samples from AD patients were measured by using a human CXCL13 kit (Quantikine; R&D Systems, Minneapolis, MN, USA). 15 CSF and 15 plasma samples obtained from age matched (among 60 and 80 years old) donors without inflammatory pathologies, which were selected as control group. Plasma was isolated by Ficoll gradient centrifugation. Blood from a patient was overlaid on Ficoll medium (GE Healthcar), centrifuged at 2700 $\times$ rpm for 20 min at 25°C and stored at -20°C. CSF samples where obtained by lumbar puncture and frozen at -20°C. Samples were allowed to thaw at room temperature and the analysis was performed according to the instructions of the manufacturer. Standard and samples were loaded undiluted. The minimum detectable dose (MDD) of human BLC/BCA-1 ranged from 0.43-3.97 pg/mL (mean 1.64 pg/mL). The MDD was determined by adding two standard deviations to the mean optical density (O.D.) value of twenty zero standard replicates and calculating the corresponding concentration.

Subject	Sex	Age	Age at onset	Diagnosis	Specimen	
					Plasma	CSF
sAD21	F	66	64	Posterior cortical atrophy	-	-
sAD22	F	67	66	Neurodegeneration	-	-
sAD23	F	69	67	AD	-	-
sAD24	M	60	56	AD	-	-
sAD25	M	61	n.a	Posterior cortical atrophy	-	-
sAD26	M	70	65	AD	-	-
sAD27	F	78	78	Atypical form of AD	-	-
sAD28	M	67	n.a	AD/CAA	-	-
sAD29	F	62	59	AD	-	-
sAD30	M	59	59	CAA	-	-
sAD31	F	76	72	AD and Vascular dementia	-	-
sAD32	M	68	66	Primary Progressive Aphasia	-	-
sAD33	M	76	72	AD	-	-
sAD34	F	71	71	AD	-	-
sAD35	F	73	70	AD/CAA	-	-
sAD36	F	65	63	Atypical form of AD	-	-
sAD37	M	80	79	AD	-	-
sAD38	M	60	58	AD	-	-
sAD39	F	71	4	AD CAA	-	-
sAD40	F	80	4	Atypical form of AD	-	-

Tab. 3.2: Clinical data available from AD patients enrolled in the ELISA assay. Dashes indicate the type of specimen used. For AD patients CSF and plasma samples were obtained from same subject.

3.2.6 Statistical analysis

Statistical analysis was carried out using the "R Project" software for Statistical Computing, version 3.5.1, with the significance level set at $p < 0.05$. Results obtained from the multiplex assay were previously tested using the Kolmogorov-Smirnov test and the Shapiro-Wilk test in order to assess the normal distribution of data. A non-normal distribution was found for all the analytes tested - except for IL-13- therefore, the non-parametric Wilcoxon test was chosen to compare data. Data were normalized according to the relative percentage of analytes (with respect to the total analytes amount). Observations were considered outlier outside 1.5 times the interquartile range above the upper quartile and bellow the lower quartile of the boxplot. Normalised data were objective for the Hierarchical Cluster Analysis (HCA). Several metrics and linkage criteria were combined in order to find the right statistical model that better fit on data analysed (Figure 3.7a).

3.3 Results

3.3.1 Neuropathological characterization of microglia in AD patients

In order to test the hypothesis that changes in the neuroinflammatory environment may contribute to the generation of different AD phenotypes, we analysed distribution and morphology of microglial cells in the frontal cortex of 15 brain samples [selected among the groups previously identified in the Neuropathology laboratory of the "Fondazione IRCCS istituto neurologico C. Besta" (AP1, AP2, AP3 and sAD1)]. by immunohistochemistry. Some dissimilarities concerning density, shape and distribution of microglial cells were observed among AD cases (Figure 3.2-3.3). In particular, the APP-A673V mutation carrier (fAD1), that is characterized by abundant amyloid deposits both in the parenchyma and in the vessels, showed a singular distribution of microglia around vessels and amyloid plaques (Figure 3.2, panels a,d,g). Microglial cells appeared in the reactive status, with hypertrophic somas and completely disappeared branches (panel j). These features are normally associated to the phagocytic state[76, 151]. Results from the quantitative analysis (panel m) indicate a strong immunoreactivity against the IBA-1 antibody and a high density of cells in the layer of the frontal cortex compared to the other AD samples. Phagocytic microglia were recognisable also in the APP-A713T (fAD2) case (Figure 3.2, panels b,e,h) who showed severe CAA and low-density parenchymal Aβ amyloid deposits in the neuropil. However, although fAD1 and fAD2 cases share the same morphological features, microglia observed in the fAD2 patient was not

organized in a specific pattern and was homogeneously distributed in all layers of the frontal cortex. Interestingly the patients carrying mutations in presenilin genes showed distinct characteristics (Figure 3.2, panels c,f,i). Despite the young age of death (age 43), microglia of mutated PS1-P117A patient was hypertrophic (panel l), condition normally associated with physiological aging[244]. The distribution was homogeneous and did not present with any specific organization but, as shown by densitometric analysis, fAD3 patient displayed the highest overexpression of IBA-1 protein and the greatest density of microglia among all the AD cases tested (panel o). Heterogeneity in microglial features was also recognisable in sporadic AD patients. Figure 3.3 shows the microglial picture of 3 cases: sAD2, sAD15 and sAD17. The first two patients showed strong microglial activation as underlined by quantification of the IBA1 immunoreactivity, the densitometry and the morphological analysis. sAD2 showed a specific organization of the microglia in a bilayer (panel a), distributed in the higher and the lower layers of the frontal cortex. Both the number and intensity of positive pixels and as well as the morphology of microglia recall those observed in the fAD1 patient even if cells appear more roundish or hypertrophic and ramified and not organized in circular structures around plaques and vessels. sAD15 exhibited a rod morphology microglia (panel k) characterized by enlarged cell body with less and thin branches departed almost perpendicularly from the longitudinal axis. Microglia were homogenously distributed, but compared to the fAD2 case, the density of positive pixel per μm2 is 30% lower. This observation suggested that, just considering the morphology and distribution and quantity, the microglial picture of AD is extremely variable and that this heterogeneity involves not only genetic forms but also sporadic patients. A clear correlation between the pattern of Aβ peptides characterizing the molecular AD subgroups (AP1, AP2, AP3 and sAD1 case) previously identified and the microglial features was not observed.

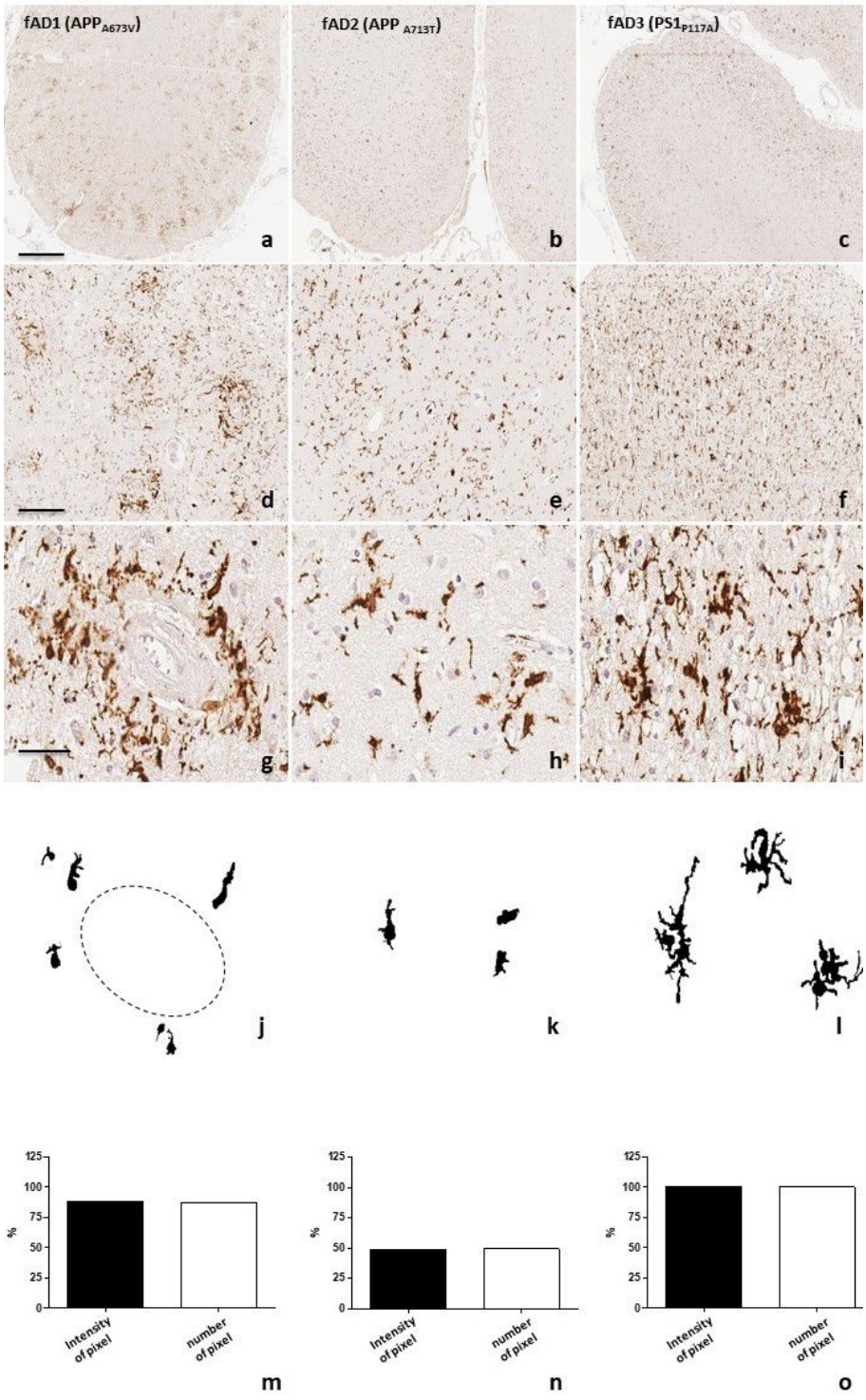

Fig. 3.2: Microglial characterization in fAD cases. (a,d,g,j,m) APPA673V; (b,e,h,k,n) APPA713V; (c,f,i,l,o) PS1P117A. Scale bar 1mm (a,b,c) 200μm (d,e,f); 50μm (g,h,i). (a,b,c,d,e,f,g,h,i) frontal cortex sections immunostained with the IBA-1 antibody. (j,k,l) morphological shape extracted and edited by imageJ software analysis. (m,n,o) quantification of IBA-1 immunoreactivity based on the intensity and number of pixels (data were normalized respect to the higher signal obtained amond AD samples).

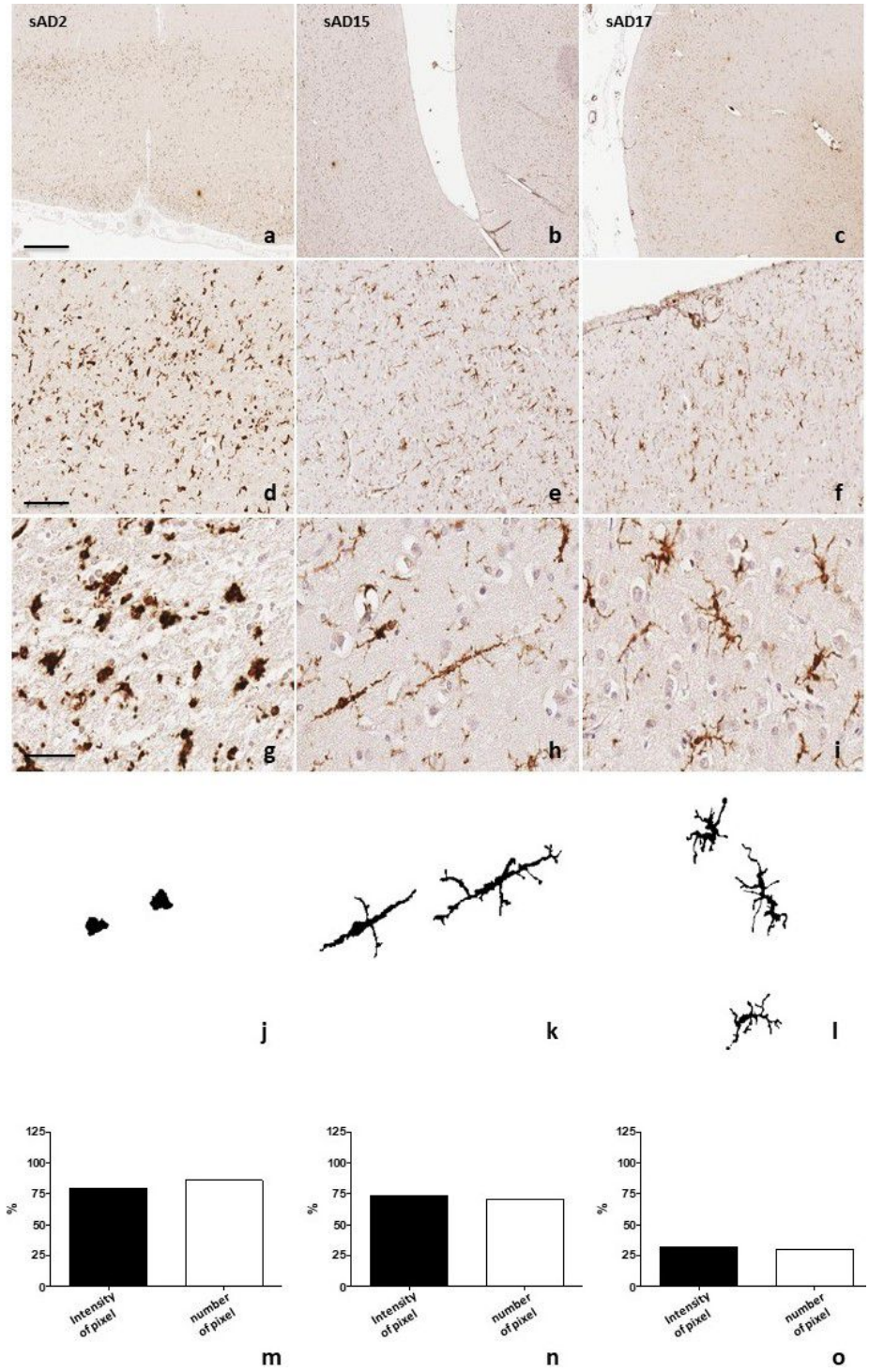

Fig. 3.3: Microglial characterization in sAD cases. (a,d,g,j,m) sAD2; (b,e,h,k,n) sAD15; (c,f,i,l,o) sAD17. Scale bar 1mm (a,b,c) 200μm (d,e,f); 50μm (g,h,i). (a,b,c,d,e,f,g,h,i) frontal cortex sections immunostained with the IBA-1 antibody. (j,k,l) morphological shape extracted and edited by imageJ software analysis. (m,n,o) quantification of IBA-1 immunoreactivity based on the intensity and number of pixels (data were normalized respect to the higher signal obtained amond AD samples).

3.3.2 Neuroinflammatory cytokines in AD patients

In order to verify if morphological changes of microglia among different AD patients are paralleled by differences in cytokines expression, we analysed a panel of pro and anti-inflammatory factors (Table 3.3), known to be expressed by microglial cells and indicated by the scientific literature as molecules potentially involved in AD pathogenesis[109]. The analysis was performed by magnetic Luminex Assay (Bio-Techne/R&D system, Minneapolis, USA) in brain homogenates of 23 AD patients and 6 aged matched control subjects.

Cytokine levels in brain samples from AD cases and controls

Because IL-6, INF-γ, IL-12, CCL-17 and CCL-10 were close or below the lowest calibrator values and not in the quantitative range of the assay for most of samples analysed, we think they should be interpreted carefully. All recovery for each calibrator was within 80%–120% of the known value (data not shown). Results showed a higher expression of overall the analytes in AD samples respect to controls, indicating that regardless the potential pro or anti-inflammatory effect of the cytokine, the neuroinflammatory process is more active in AD patients than controls (Figure 3.4). In particular, the highest levels were found in AD patients for IL6 (2.5-fold increase respect to controls). CCL2, CCL5, CXCL9, CXCL10 and MMP8 were all above the 1.5-fold increase in comparison with control samples. IL1 was found at lower levels in AD cases than in controls.

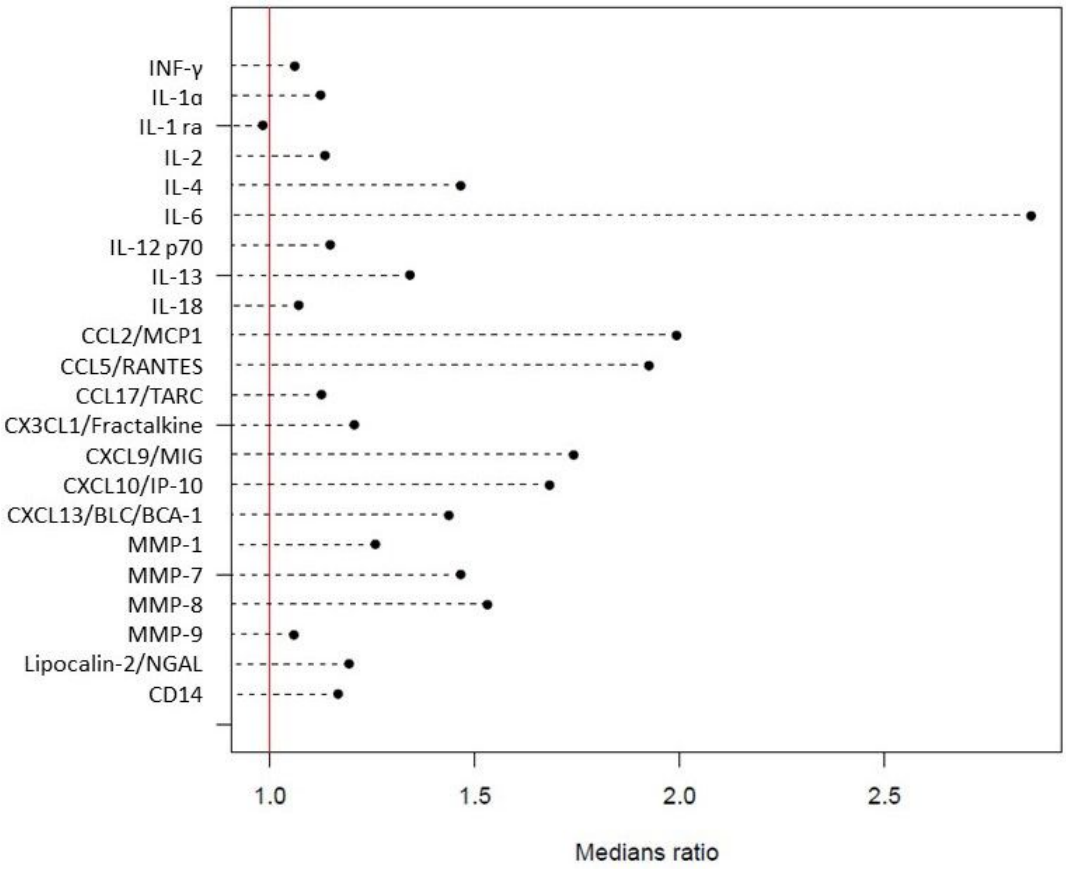

Fig. 3.4: AD Vs CTRLs median ($\mu 1/2$) ratio. (ADs $\mu 1/2$)/ (CTRLs $\mu 1/2$) expresses how much AD samples are elevated respect controls for each cytokine. Ratio = 1 indicates no differences among groups; ratio < 1 indicates that controls are higher than ADs; a ratio > 1 means that AD cases have a higher expression of cytokine respect to controls.

Statistical analysis showed significantly higher concentrations of IL-4 (p = 0,0334), IL-6 (p = 0,0288), IL-13 (p = 0,0060), CCL-17 (p = 0,0376) and CXCL13 (p = 0,0009) in AD samples (Table 3.3 and Figure 3.5 panels q-v). Interestingly, CXCL13, the most significant analyte detected in brain homogenates (Figure 5, panel v), has never been directly associated to AD, therefore we decided to further analyse this chemokine in biological fluids from AD patients, following the hypothesis that it could represent a biomarker relevant for AD.

Analytes	**Cases** $\mu_{1/2}$	**Controls** $\mu_{1/2}$	**Rate**	**P-values**	
INF-γ	51,06	48,13	1,061	0,6278	
IL-1α	9,74	8,67	1,123	0,1249	
IL-1 ra	695,28	707,43	0,983	0,5812	
IL-2	336,49	296,64	1,134	0,0559	
IL-4	**73,53**	**50,19**	**1,465**	**0,0334**	*
IL-6	**6,03**	**2,11**	**2,858**	**0,0288**	*
IL-12 p70	142,32	124,08	1,147	0,3887	
IL-13	**690,69**	**514,71**	**1,342**	**0,0060**	**
IL-18	66,68	62,27	1,071	0,0795	
CCL2/MCP1	108,45	54,43	1,993	0,0951	
CCL5/RANTES	29,39	15,27	1,925	0,0559	
CCL17/TARC	**89,11**	**79,17**	**1,126**	**0,0376**	*
CX3CL1/Fractalkine	7509,91	6225,31	1,206	0,3841	
CXCL9/MIG	859,89	493,92	1,741	0,1870	
CXCL10/IP-10	10,91	6,49	1,682	0,1418	
CXCL13/BLC/BCA-1	**62,37**	**43,41**	**1,437**	**0,0009**	***
MMP-1	228,83	181,96	1,258	0,0544	
MMP-7	449,83	306,91	1,466	0,0052	**
MMP-8	5383,96	3516,87	1,531	0,2118	
MMP-9	11933,74	11272,82	1,059	0,8135	
Lipocalin-2/NGAL	5340,23	4475,46	1,193	0,1575	
CD14	10439,33	8948,05	1,167	0,1744	

*Tab. 3.3: The median ($\mu_{1/2}$) and the rate of measurements (pg/mL) for the detectable analytes in AD versus control. Values in BOLD underline the statistically significant differences between the two groups of samples. 0,01<*p<0,05, 0,001<**p<0,01, ***p<0,001.*

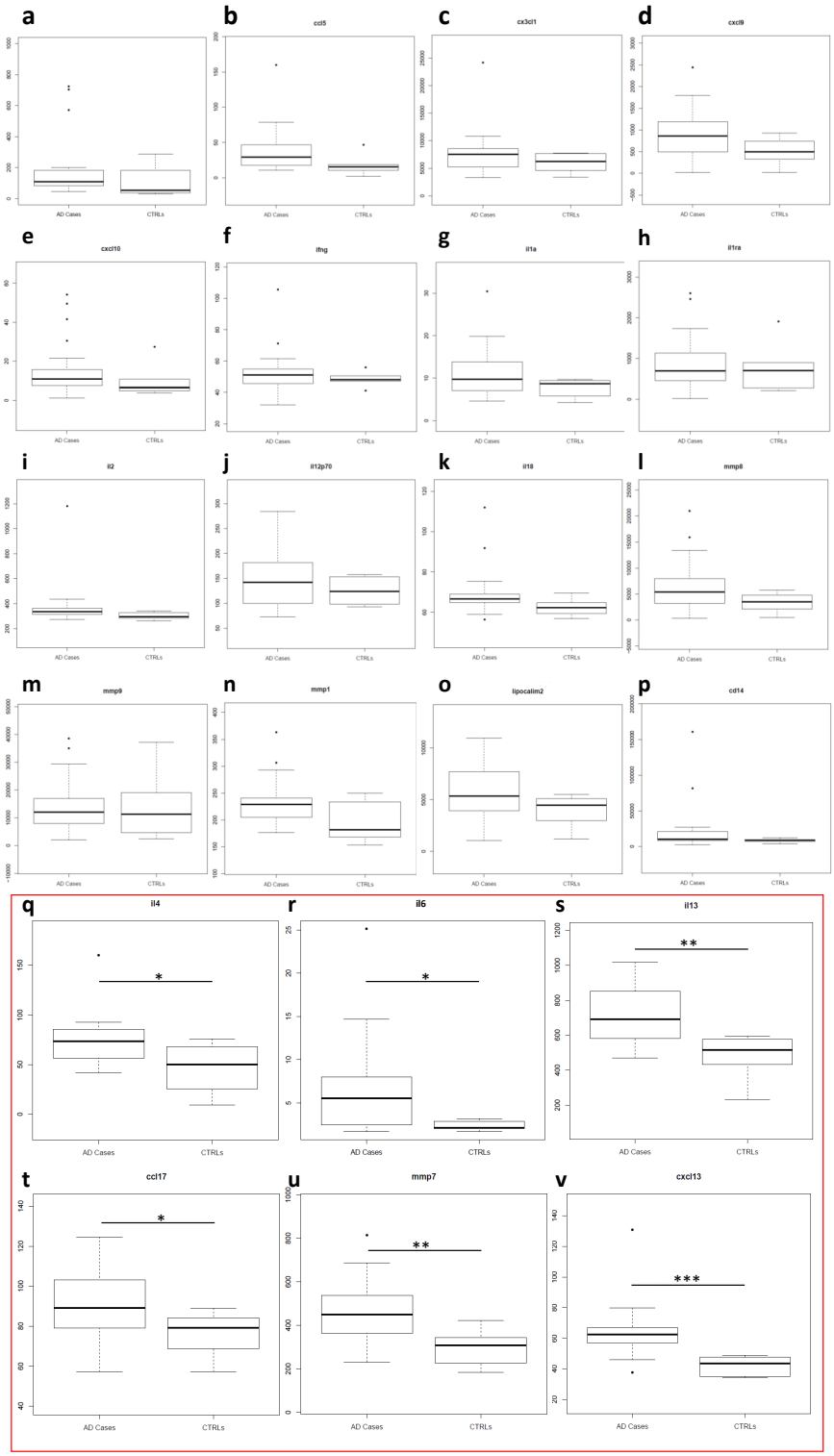

Fig. 3.5: Comparison between AD and control samples for each cytokine, chemokine, MMP and innate factors. Boxplots for CCL2 (a), CCL5 (b), CX3CL1 (d), CXCL9 (d), CXCL10 (e), INF-γ (f), IL-1a (g), IL-1ra (h), IL-2 (i), IL-12 p70 (j), IL-18 (k), MMP-8 (l), MMP-9 (m), MMP-1 (n) Lipocalin2 (o), CD14 (p). Boxplots of the most significant analytes, IL-4 (q), IL-6 (r), IL-13 (s), CCL-17 (t), MMP-7 (u), CXCL13 (v) are highlighted in red. Figure (a) CCL2 and (r) IL-6 are presented without out-layers to better appreciate the difference between AD cases and controls.

3.3.3 Correlation between AD molecular profiles and neuroinflammatory clusters

To check a possible correlation between the molecular AD subgroups previously identified and the levels of neuroinflammatory factors quantified by multiplex assay, we performed a hierarchical cluster analysis. Data from multiplex assay were normalized and clustered in order to obtain different subgroups of AD patients characterised by different expression of neuroinflammatory factors. The HCA sets yielded a dendrogram based on the Euclidean distance among groups (Figure 3.6). The higher is the value expressed in ordinate, the greater is the difference between two clusters. The Thorndike's procedure[256] was applied to establish the appropriate number of clusters (CL1, CL2, CL3, CL4). In our case, we were able to classified data into 4 clusters that identified 4 distinct neuroinflammatory profiles within the AD cohort. Results showed no clear correspondence between "neuroinflammatory subsets" of AD and AP subgroups based on differences in Aβ species. We obtained the same results even using different cluster analysis (Figure 3.7).

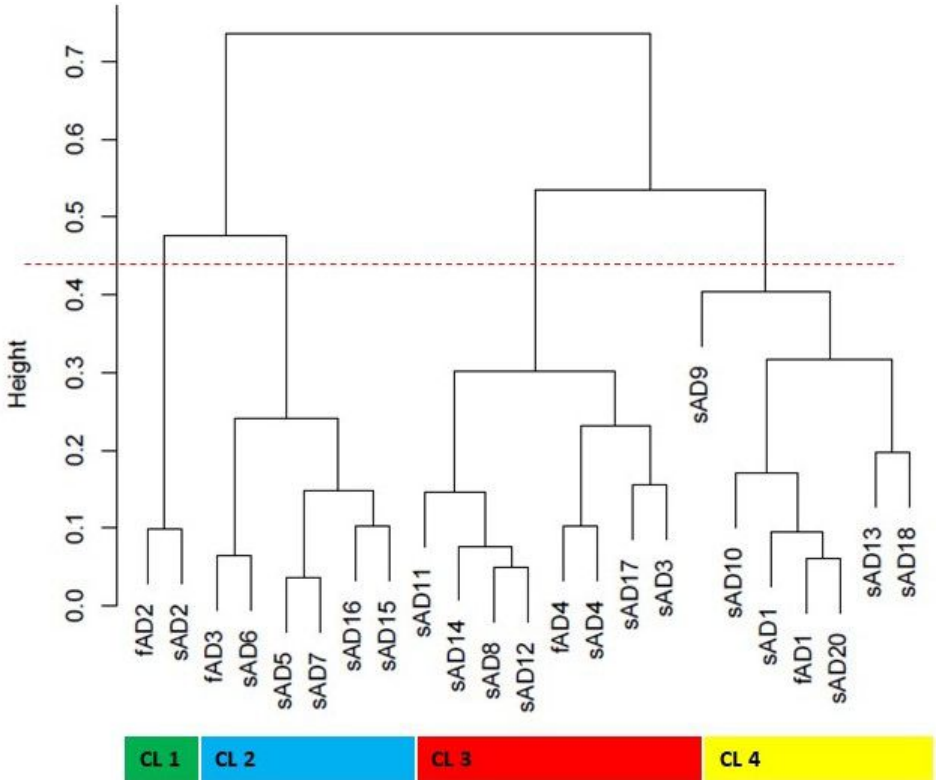

Fig. 3.6: Classification of AD patients according to the differential expression of neuroinflammatory factors. Data from multiplex assay have been normalized and divided according to the Hierarchical cluster analysis (HCA), where the abscissa represents individual AD patient and the ordinate corresponds to the linkage distance measured by Euclidean distance. The dashed red line denotes the cut off for four clusters (CL), numbered one through four and underlined by colours green, light blue, red and yellow respectively. Each cluster represents a different subgroup of AD patients characterised by different expression of neuroinflammatory molecular factors.

a

Metric	Linkage criteria	Cluster
Euclidean	Complete	Cluster 1
Euclidean	Single	Cluster 2
Euclidean	Average	Cluster 3
Manhattan	Complete	Cluster 4
Manhattan	Single	Cluster 5
Manhattan	Average	Cluster 6

b

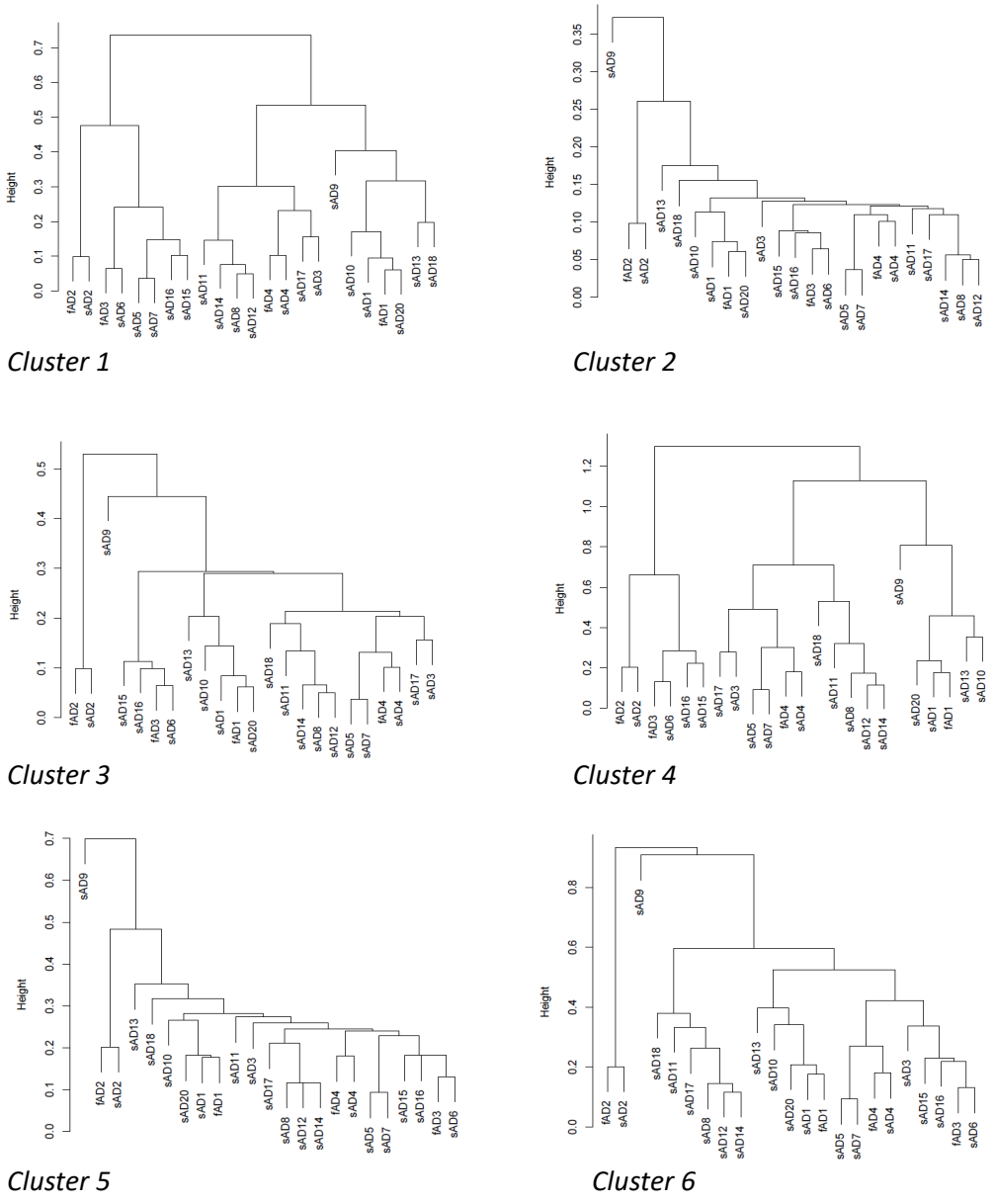

Fig. 3.7: Hierarchical Cluster Analysis. Table in (a) shows the combination of metrics and linkage criteria used for generate cluster dendograms.

3.3.4 Analysis of chemokines in CSF and plasma from AD patients and controls

CSF and plasma are useful and more accessible biological specimens to detect peripheral biomarkers in AD. In order to evaluate CXCL13 as potential neuroinflammatory biomarker in AD, we enrolled both patients affected by AD and control subjects and compared CXCL13 levels obtained by ELISA assay. We measured the chemokine concentrations (pg/mL) in CSF of 17 AD patients and 16 subjects with no inflammatory-related pathologies. Three control subjects were excluded from the analysis because they resulted affected by inflammatory pathologies. Results showed significantly higher concentration of CXCL13, p-value 0,04207, in AD patients than controls (Figure 3.8). We also noted that levels of this chemokine in CSF were extremely low (under the last point of the standard curve) and compare to those quantified in the brain homogenate, it appeared 20 and 16 times lower in patients and controls respectively.

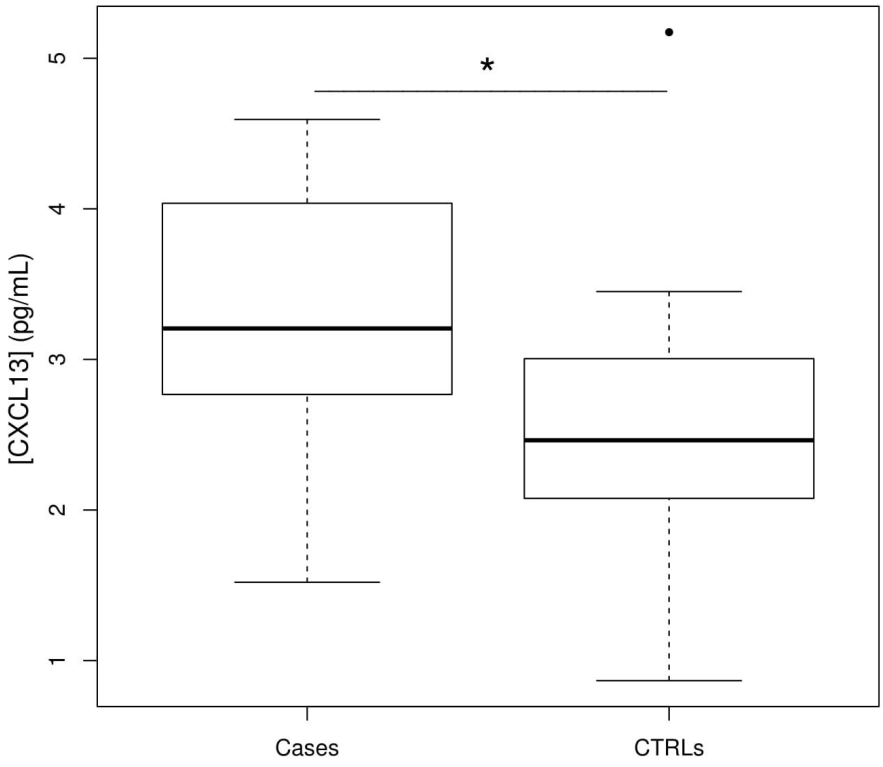

Fig. 3.8: Concentrations of CXCL13 in CSF of AD patients and control subjects. Boxplots were designed without outlayers (three samples were excluded from AD group and one from controls). Observations were considered outlier outside 1.5 times the interquartile range above the upper quartile and bellow the lower quartile of the boxplot.

CXCL13 concentrations were then measured in plasma samples. For this study, we enrolled 20 AD patients (17 corresponded to those used for the CSF analysis) and 20 controls subjects carefully selected without inflammatory pathologies. This time, the chemokine values analysed were much higher than those observed in CSF samples and fell within the standard curve. However, although there was a slight increase in CXCL13 in AD patients compared to controls, no statistically significant difference (p-value 0.1022) was found between groups (Figure 3.9). Nevertheless, these data showed a trend towards an increase of CXCL13 in AD patients in line with that observed in brain homogenates and CSF specimens. Probably, the low number of samples used in this experiment was insufficient to appreciate a process so distant to its origin with significantly statistical differences.

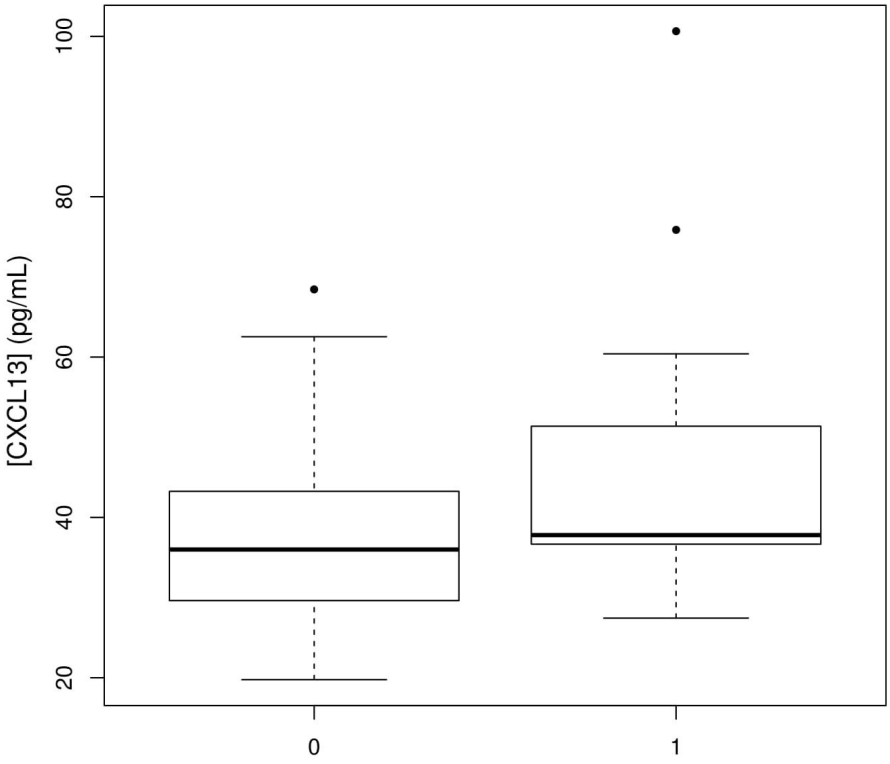

*Fig. 3.9: Concentrations of CXCL13 in Plasma of AD patients and control subjects. Boxplots were designed for all samples, 20 AD patients and 20 controls subjects. No statistically differences were found: *P-value 0.1022.*

3.4 Discussion

Neuroinflammation is a fascinating and still largely unexplored aspect of AD. The high complexity and variability of this process may contribute to both the pathogenesis and the generation of different AD phenotypes. Supported by findings that mutations in genes encoding for immune receptors, such as TREM227 and CD338, increase the risk to develop AD, neuroinflammation acquired great importance in the pathogenesis of the illness. Moreover, previous gene expression studies suggested that the neuroinflammatory pathway may be a source of heterogeneity in AD. According to the study of Sudduth and colleagues, the apparent polarization of the neuroinflammatory state to M1 or M2a in early AD could influence the response to several therapeutic agents. Moreover, they identified many proteins in serum that are predictive of the brain neuroinflammatory state and that could be used to stratify groups in clinical trials[249]. Following this view, the second part of this study was aimed at investigating on the involvement of some cellular and molecular players of neuroinflammation - i.e. microglial cells and several cytokines - in the chain of pathogenic events leading to AD and their possible implication in the occurrence of the disease under distinct phenotypes. The study was focused in particular on microglia because their activation results in several neuroinflammatory subtypes of these cells which could modulate the severity of the disease[35, 241]. We started from the neuropathological characterization of microglial cells, choosing the IBA-1 antibody as marker for the detection of both the quiescent and the activated microglia. We found that microglial cells are differently represented within the brain of AD patients and that such differences concern morphology, distribution, density and intensity of the activation in brain tissue. These features suggest that the microglial picture of AD is extremely variable among both sporadic and genetic forms of the disease, since microglial cells are able to organize and distribute differently within the parenchyma generating different patterns of activations in AD brains. The morphology reflects the functional state of these cells, indicating a change from the quiescent to an activated state. Coherently with the late stage of the disease, microglia appeared activated for almost all cases analyzed in this study – which are mostly in the advanced Braak stages -, even if we noted some samples where the IBA-1 signal was very low. We also observed high variability among within the same area, especially between the grey and the white matter. Indeed, in the white matter cells appeared indiscriminately activated. These findings confirmed previously reported data on the morphological and regional variability of microglial activation[72] and may also be implicated in the selective vulnerability of some brain areas to the AD pathology[90]. A clear correlation between the pattern of Aβ peptides characterizing the molecular AD subgroups (AP1, AP2, AP3 and sAD1 case) previously identified and the microglial features was not observed. Furthermore, we did not find any correspondence between the microglial pattern and neither the

ApoE haplotype nor the clinical picture of the patients analysed in this study. The low number of cases analysed in the study limited the possibility of studying in depth the correlations between clinical-pathological variability of AD patients and the different patterns of microglial activation which were founds in the cohort of AD cases. So, the relevance of the variances in microglial activations in the phenotypic heterogeneity of AD remains undetermined and needs to be addressed by further studies on larger cohorts. However, morphological features alone are not sufficient to identify specific subsets of neuroinflammation in AD, thus we moved the analysis to the molecular level with the purpose of analysing the chemokines released by microglial cells during their activation state. Several studies have already proved the implication of inflammatory molecules in AD pathology. For example, elevated levels of TNF-α and IL-6 were found in the serum and in brain tissue of AD patients, respectively[73, 11]. Interestingly, these differences can be used to stratify AD patients in neuroinflammatory phenotypes. Following an approach proposed by Chen and colleagues[29] consisting of a multiplex analysis of the differential expression of neuroinflammatory molecules secreted by microglia, we firstly observed that the overall levels of the microglia-derived neuroinflammatory factors are significantly higher in AD samples than controls regardless of their pro or anti-inflammatory effect. In particular, the abundance of pro-inflammatory cytokines is explained by the state of chronic inflammation caused by the continuous overproduction of pathogenic molecules that is characteristic of AD. Moreover, the brain samples used in this study come from AD patients in advanced stage of the disease (Braak stage for almost patients V-VI). On the other hand, the presence of anti-inflammatory factors is not surprising taking into account that the anti-inflammatory pathways may reflect a defensive response of the brain tissue to an excessive inflammatory reaction against misfolded proteins (i.e., Aβ and Tau). This view agrees with previous studies that proved that the stimulation of some pro-inflammatory signalling pathways leads to strong neuroinflammation able to reduce amyloid plaque pathology in AD mouse models[235, 77]. This inflammatory process may be detrimental if inflammation is unable to find a resolution. Interestingly, as for AD, time is a risk factor for the inflammatory process that under chronic stimulation became deleterious. Secondly, we tried to group AD patients based on the relative percentage of inflammatory molecules expressed, identifying four clusters (named CL1-CL4). Only two AD patients compose CL1, which is characterized by the lowest levels of all the molecules analysed except for CD14 that appeared the highest among all clusters. In microglia, CD14 interacts with Aβ fibrils contributing to their phagocytosis[154]. These data are in accordance with the low amyloid burden and the classical phagocytic morphology observed in the fAD2 and sAD2 patients. CL2 and CL3 showed similar anti-inflammatory features. However, these features result from two different behavioural: The CL2 showed high expression of several anti-inflammatory cytokines like IL-1a, IL-1ra, IL-13, IL-18 and CCL5, while CL3 displayed low levels of pro-inflammatory cytokines like IL-6 and CCL2. CL4 showed an opposite profile respect to the one observed

in the CL1. Among all clusters, it is characterized by the highest levels of all the molecules analysed except for CD14 that resulted very low. As for microglia, we compared neuroinflammatory profiles with the molecular AD subgroups previously identified in our laboratory but we did not find any substantial correlations. These data suggest that the cytokines released by microglial cells are likely not directly implicated in the generation of the molecular subgroups of the disease which were defined based on the Aβ content of amyloid assemblies. During the characterization of the neuroinflammatory profiles we came across to an interesting finding. We noted that levels of CXCL13, a chemokine never associated with AD, resulted to be more expressed in brain homogenates, CSF and, to lesser extent, in plasma samples of AD patients respect control subjects. CXCL13, also known as B lymphocyte chemoattractant (BLC) protein, is a chemokine selectively chemotactic for B cells belonging to both the B-1 and B-2 subsets, and elicits its effects by interacting with the chemokine receptor CXCR5 expressed by microglia and T and B lymphocytes. High CSF CXCL13 levels were demonstrated to correlate with increased B cell recruitment in CNS in a range of human disorders such as lymphoma, Lyme disease and multiple sclerosis (MS)[218, 219, 232]. In patients affected by MS, CXCL13 secreted by microglia, is involved in the recruitment of Th1, Th17 and B cells[114] and more recent reports demonstrated that CXCL13 could be a useful marker in the diagnosis of MS[5]. Moreover, in mice with experimental autoimmune encephalomyelitis (EAE), an important animal model of MS, it was demonstrated that CXCL13 and its related pathways actually contribute to disease pathogenesis and are not simply markers of neuroinflammation. In AD, there are no evidence supporting a role of CXCL13 in the pathogenesis of the disease. However, several studies demonstrated the involvement of Th1 and Th17 cells in the AD-related neuroinflammatory process. For example, Zhang and colleagues hypothesized that Th17 cells are directly responsible for neuronal cell death through the interaction of Fas and FasL transmembrane proteins[286]. On the other side, B cells are involved in AD through the production of autoantibodies. A vast part of autoantibodies is believed to be produced in response to the toxic aggregated forms of Aβ, including oligomers and protofibrils both in periphery and in CNS[60, 250]. In this sense, the study of CXCL13 could provide new insights into the molecular mechanisms of the adaptive immune response in AD. The data provided by this study about the overrepresentation of CXCL13 in brain tissue and biological fluids of AD patients nominate this chemokine as a potential novel neuroinflammatory biomarker for AD. To test this hypothesis, additional studies on larger cohorts of patients and controls are needed.

3.5 Conclusions

Given the multifactorial nature of AD, which can display variable clinico-pathological pictures, the studies described in this thesis were carried out with the aim of identifying the most important players of the bio-molecular heterogeneity of the disease The project was focused on two specific aspects involved in AD pathogenesis:(i) the Aβ peptide and (i) the neuroinflammatory environment. The experimental plan was designed to explore the contribute of these two factors in the generation of a "bio-molecular diversity" paralleling the clinico-pathological heterogeneity of AD, and to identify possible correlations between the heterogeneity at molecular level and the diversity of clinical and neuropathological pictures of the disease. Mass spectrometry studies showed that is possible to isolate different Amyloid Profiles (AP) among AD patients corresponding to different molecular subtypes of the disease. The different profiles are distinguished on the basis of the presence and the relative quantity of different Aβ isoforms in the Aβ assemblies accumulating in brain tissue. These distinct assemblies have actually different biological properties that could, at least partially, explain the phenotypic variability of AD. Indeed, they:

- Show different aggregation kinetics;
- Display distinct seeding abilities and seed-substrate affinities;
- Are characterized by a different degree of resistance to digestion with endogenous proteases;
- Have different attitudes to induce amyloidosis when injected in animal models.

The correlation found among aggregation kinetics, resistance to proteases degradation, seeding activity in vitro, and capability to trigger beta-amyloidosis having distinctive features in animal models supports the hypothesis that the variability of AD phenotypes may come, at least in part, from the biochemical composition of Aβ aggregates, mainly from the content of Aβ isoforms which are recruited in the aggregation pathway leading to the formation of amyloid assemblies. The neuropathological study on microglia revealed a high variability related to the morphology, density and distribution of these cells within the brain of AD patients. Morphological changes reflect distinct functional states of activation that lead microglia to release several pro- and anti-inflammatory molecules which in turn influence the development and the heterogeneity of the disease. Overall levels of neuroinflammatory factors analyzed by multiplex assay resulted to be significantly higher in AD samples than controls regardless of their pro or anti-inflammatory effect. These data indicate the presence of a neuroinflammatory process within the brain of AD patients that

can be relevant for the pathogenesis of AD and for the occurrence of the disease under different phenotypes. Moreover, the data indicated that is possible to isolate distinct neuroinflammatory subgroups of AD by analyzing the relative quantity of different inflammatory molecules. However, no complete overlap between AD subgroups identified by mass spectrometry and neuroinflammatory profiles was found. Interestingly, we found statistically significant differences in the levels of the CXCL13 in brain and CFS - and, to a lesser extent, in plasma - of AD patients in comparison with controls. These findings may lead to unveil a novel potential biomarker for neuroinflammation in AD, but further investigations are required to fully characterize the implications of this chemokine in AD pathology and to explore its use as a biomarker. The work carried out within this project focused on two aspects potentially involved in the phenotypic heterogeneity of AD. However, we are aware of the multitude of factors that could play a role in this phenomenon and that the full knowledge of the molecular basis of phenotypic diversity in AD needs additional studies on larger cohorts of patients. As a concluding remark, it is important to underline the relevance and utility of these studies, because, in the future, they could:

1. Lead to a reclassification of AD based on molecular subtypes;

2. Enable the identification of effective biomarkers useful for the identification of distinct disease phenotypes;

3. Help clinicians to make a more accurate and early diagnosis of the disease;

4. Help patients to receive clearer prognosis;

5. Open the way to the development of more tailored therapies for different phenotypes of AD.

The latter objective is particularly important, considering that the failure of the therapeutic strategies adopted until now for AD may be due, at least in part, to the existence of atypical forms of the disease, which show different resistances to pharmacological treatments.

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Declaration

I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the Degree of Doctor of Neuroscience.

Milan, October, 2018

Stefano Sorrentino