Introduction

1. Alzheimer's Disease

Alzheimer's disease (AD) is a chronic and fatal neurodegenerative disease and the most common form of dementia, affecting ca. 60-70% of 55 million people worldwide (2023 Alzheimer's disease facts and figures). Furthermore, researchers from the Institute for Health Metrics and Evaluation at the University of Washington School of Medicine, as reported at AAIC 2021, anticipate arise in dementia cases, estimating that the number of affected people will nearly triple to exceed 152 million by 2050 (2021 Alzheimer's disease facts and figures; World Health Organization, 2021) becoming one of the most expensive, lethal, and burdening diseases worldwide (Alzheimer Europe, 2020).

AD is characterized by well-defined pathophysiological mechanisms (Alzheimer *et al.* 1995; Blessed, Tomlinson and Roth 1968; Glenner and Wong 1984; De Strooper and Karran 2016; Guo *et al*. 2020; Kim *et al*. 2023) leading to memory loss and difficulties with thinking, language and problem-solving skills. Neuropathologically, the AD is characterized by the presence of extracellular neurotic plaques, arising from the accumulation of the amyloid-beta (Aβ) peptide, and intracellular neurofibrillary tangles, composed of the hyperphosphorylated microtubule-associated tau protein (Glenner and Wong 1984; Kosik, Joachim and Selkoe 1986) as schematically represented in Figure 1.

These deposits are thought to play a crucial role in neurodegeneration, leading to several cytotoxic pathways such as excessive stimulation of neurotransmitter receptors, loss of calcium homeostasis, inflammation and depletion of energy and neuronal factors (Li S. *et al*. 2009; Heneka *et al*. 2015; Popugaeva, Pchitskaya and Bezprozvanny 2018; Butterfield and Halliwell 2019). However, recent studies have shown that the deposition of amyloid in the brain represents the first sign of the disease, preceding the clinical diagnosis of dementia by up to 20 years (Jack *et al*. 2013b; Jansen *et al*. 2015; Winblad *et al*. 2016; Vermunt *et al*. 2019). Indeed, AD

Liliana Napolitano, liliana98.napolitano@gmail.com, 0009-0004-3087-286X

Referee List (DOI 10.36253/fup_referee_list)
FUP Best Practice in Scholarly Publishing (DOI 10.36253/fup_best_practice)

Book References DOI 10.36253/979-12-215-0993-9.references

presents a pre-symptomatic period that may last a few years, if not over a decade, before cognitive symptoms (Sperling *et al*. 2011; Villemagne *et al*. 2013), a mild cognitive impairment (MCI), which is characterized by a detectable cognitive deficiency without impact of daily activities (Albert *et al*. 2011; Brodaty *et al*. 2013) and regarded as an intermediate, transitional period from normal cognition to AD, when the disorder is evident as a form of dementia (McKhann *et al*., 2011; Albert *et al*. 2011; Brodaty *et al*. 2013).

The aforementioned phases are characterized by several clinical symptoms. Indeed, different studies at this time showed that episodic memory loss (*i.e*., amnesia) is usually the earliest and most prominent aspect of the AD dementia (Walsh and Selkoe 2004; Bäckman *et al*. 2005; Sperling *et al*. 2011; Weintraub, Wicklund and Salmon 2012).

These findings were consistent with neuropathologic studies that showed extensive AD pathology occurring earliest in medial temporal lobe (MTL) structures (*e.g*., hippocampus, entorhinal cortex), and responsible for episodic memory loss (Hyman *et al*. 1984; Bondi, Edmonds, and Salmon 2017). Furthermore, a deficit in language skills (*i.e.*, aphasia) was observed relatively early during AD, with deficits in confrontation naming, verbal fluency (especially in semantic categories), and a reduced ability to recall learned facts (*e.g.*, the number of days in a year) (Hodges, Graham and Patterson 1995; Nebes 1989; Bondi, Edmonds, and Salmon 2017). In addition, deficits in "executive" functions responsible for the simultaneous mental manipulation of information, concept formation, problem solving, and cue-directed behavior, have been found to develop during AD (Bondi *et al.* 1994; Lafleche and Albert 1995; Perry and Hodges 1999; Bondi, Edmonds, and Salmon 2017). The gradual decline in cognitive function, therefore, contributes substantially to the loss of autonomy and reduced quality of life suffered by patients (Heyman *et al*. 1996; Whitehouse *et al*. 1997; Corey-Bloom 2002).

1.1 Etiology and genetics

AD can be classified into early-onset and late-onset AD. Early-onset AD accounts for approximately 1– 6% of all cases and manifests roughly between 30 and 60 years of age. The late-onset form, accounting for around 90% of cases, has an age of onset later than 60 years of age (Revi 2020). Besides the age of onset of symptoms, the early and late forms of AD differ in many clinical, neuropsychological, neuropathological and neuroimaging variables (Mendez 2017). Indeed, the existence of AD "variants", as posterior cortical atrophy (PCS) (Hof *et al*. 1997; Renner *et al.* 2004) and a frontal variant of AD (Johnson *et al.* 1999) has complicated the clinical and neuropsychological differentiation of AD from other neurodegenerative diseases that may have a different underlying focal pathology such as Frontotemporal Lobe Dysfunction (FTLD), Dementia with Lewy Bodies (DLB), or Primary Progressive Aphasia (PPA) (Bondi, Edmonds, and Salmon 2017). In addition, genetics play a crucial role in AD, with a heritability of 58–79% for late-onset disease and over 90%

for the early-onset one, respectively (Sims, Hill and Williams 2020). Dominantly inherited mutations in amyloid-β precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) are associated with early onset AD (Karch, Cruchaga and Goate 2014).

APP is located on chromosome 21q21. Dominant mutations in this gene account for approximately 14% of early onset autosomal dominant cases of AD, with more than 30 mutations described (Guerreiro, Gustafson and Hardy 2012; TCW and Goate 2017). Two recessive APP mutations, A673V and E693Δ, were also reported to cause early onset AD (Guerreiro, Gustafson and Hardy 2012). The majority of mutations in APP cluster in the region that is adjacent to or within the Aβ domain; however, early genetic studies only sequenced the exons encoding the Aβ sequence (16 and 17), leaving the possibility that variants may exist elsewhere in APP, thus causing or increasing the risk for AD (Karch, Cruchaga and Goate 2014). PSEN1 is located on chromosome 14q24.3, while its homologue, PSEN2, is located on chromosome 1q31-q42. PSEN1 and PSEN2 are structurally similar integral membrane proteins containing nine transmembrane domains with a hydrophilic intracellular loop region (Guerreiro, Gustafson and Hardy 2012) and critical components of the γ-secretase complex, which cleaves APP into Aβ fragments (Karch, Cruchaga and Goate 2014).

In late-onset AD, the strongest genetic risk factor is the apolipoprotein E (APOE) genotype. APOE, which encodes the brain's major cholesterol transporter, has three common alleles: ε2 (8.4% estimated allele frequency in the population), ε3 (77.9%), and ε4 (13.7%) (Liu *et al.* 2013). APOE ε4 contributes to AD risk via a multitude of mechanisms, including enhanced aggregation and decreased clearance of the Aβ peptide, increased tau phosphorylation, neuronal network hyperexcitability, reduced glucose metabolism, impaired vascular and mitochondrial function (Liu *et al.* 2013).

Genome-wide association studies have identified more than 20 additional common genetic variants that modify the risk of late-onset AD (Carmona, Hardy and Guerreiro 2018). These genes converge in biological pathways involving lipid metabolism, innate immunity, and endocytosis; the effects of each one on AD risk is small (odds ratios of approximately 0.8 to 0.9 for protective alleles and 1.1 to 1.2 for risk alleles) and not clinically meaningful (Rabinovici 2019). The genetic landscape of AD is entirely reported in Figure 2.

2. Protein misfolding

Protein misfolding diseases is believed to be the primary cause of several neurodegenerative diseases, including AD. These diseases are characterized by a generic failure of the proteostasis network (PN), that is composed of the protein translation machinery (PTM), molecular chaperones (MC), the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP), that together cooperate to maintain proteins in a soluble non-aggregated state (Brehme *et al.* 2014; McKinnon and Tabrizi 2014; Martinez-Lopez, Athonvarangkul and Singh 2015). Schematic representation of altered proteostatsis system in Figure 3.

2.1 Protein translation machinery (PTM)

As the foundation of translational control, PTM directly influences the quality and quantity of newly synthesized peptides, which in turn affects cellular adaptation (Jackson, Hellen and Pestova 2010; Yuan, Zhou and Xu 2024). Once mRNA has entered the translation pool, the remaining steps are completed by the translation machinery, which is primarily composed of ribosomes, transfer RNA (tRNA), and translation factors (TrFs), including initiation factors (eIFs), elongation factors (eEFs), and termination factors (eRFs) (Jackson, Hellen and Pestova 2010).

In MCI and AD dementia, several studies showed altered composition of ribosomes, abnormal expression levels of certain transcription factors, and impaired protein synthesis in cortical areas (Sajdel-Sulkowska and Marotta 1984; Ferrer 2002; Ding *et al.* 2005; Li *et al.* 2004; Hernández-Ortega *et al.* 2016). The main modifications implicated increased RNA oxidation as evidenced by increased 8-hydroxyguanosine immunoreactivity, reduced rRNA levels and capacity of isolated polyribosomes to incorporate S35 methionine into protein (Ginsberg *et al.* 1998; Nunomura *et al.* 2001; Ding *et al.* 2005; Hernández-Ortega *et al.* 2016). Furthermore, other studies have shown that the nuclear organizer region (NOR) surface/total nucleus surface is reduced (Nyhus *et al.* 2019; Brooks 2024), and that the rDNA promoter is hyper-methylated in AD, thus suggesting epigenetic silencing of rDNA at very specific times of AD progression (Pietrzak *et al.* 2011). Overall, these results indicate that many processes related to protein production, from the nucleus and nucleolus to the ribosome, are affected AD brain.

2.2 Molecular chaperones (MC)

Chaperones are crucial for maintaining cellular proteostasis by aiding in protein folding, preventing aggregation, and facilitating degradation of misfolded proteins (Chiti and Dobson 2017; Scalia *et al*. 2021; Kravats,Wickner and Camberg 2022). Cellular stress, arising from factors like heat shock or oxidative stress, increases the burden of misfolded proteins, leading to elevated chaperone synthesis as a protective response (Kravats,Wickner and Camberg 2022). Chaperones are categorized based on sequence homology and molecular weight into distinct families, such as Hsp40, Hsp60, Hsp70, Hsp90 and others, each contributing uniquely to the complex network of proteostasis maintenance (Kim *et al*. 2013; Camberg *et al*. 2013).

In pathological stages, chaperones and co-chaperones lose their ability to fold and degrade by the lysosome, and proteins such as tau and Aβ accumulate in the brain tissue of AD patients (Gorantla and Chinnathambi 2018; Batko *et al*. 2024). Studies have shown that Hsp90, an essential heat shock protein (Hsp) (Taipale, Jarosz and Lindquist 2010; Batko *et al*. 2024), plays a crucial role in AD. Alonso and coworkers, have proven that Hsp90 may regulate tau phosphorylation and dephosphorylation (Alonso *et al*. 2018). Moreover, certain co-chaperones like

Protein Phosphatase 5 (PP5), Cell Division Cycle 37 (Cdc37), and Calcyclin Binding Protein/Siah-1 Interacting Protein (CacyBP/SIP) contribute to tau dephosphorylation, which may help to prevent its aggregation (Jinwal *et al*. 2011; Wasik *et al*. 2013). However, the activity or proper localization of these enzymes often declines or is disrupted with age, potentially disrupting this protective mechanism. Conversely, the co-chaperone C-terminus of Hsc70-interacting protein (CHIP) facilitates tau degradation through ubiquitination, and its dysfunction results in tau accumulation (Dickey *et al*. 2008). Another important chaperone in AD context is Hsp70. Hsp70 enhances the Aβ clearance through the upregulation of Insulin Degrading Enzyme (IDE) and Transforming Growth Factor-β1 (TGF-β1) (Lu *et al*. 2014). IDE is an Aβ-degrading enzyme that increases the clearance of Aβ. TGF-β1 is a key cytokine involved in inflammatory response regulation in the brain and has been suggested to slow down AD progression. Precisely, TGF-β1 leads to Aβ clearance through activation of phagocytic microglia (Lu *et al*. 2014).

These findings suggest that age-related changes in Hsp90 co-chaperone and Hsp70 function may play a significant role in the development of neurodegenerative diseases like AD, where tau pathology is a key hallmark.

2.3 The ubiquitin-*p*roteasome system (UPS) and the autophagy-lysosome pathway (ALP)

The ubiquitin proteasome system (UPS) and autophagy-lysosome pathway (ALP) are predominant methods of proteolysis in cells. Many small proteins that have completed their cellular functional roles or are aberrantly folded are linked to a small protein, called ubiquitin, for degradation by the 26S proteasome, a multi-subunit proteolytic complex, being eliminated through the UPS pathways (Kleiger and Mayor 2014). In contrast, large protein aggregates and cellular organelles undergo degradation by the ALP (Bento *et al*. 2016). Failure of these protein degradation systems is associated with disrupted proteome homeostasis as well as development of pathogenic diseases as AD, Parkinson's Disease (PD), Huntington's Disease and other neurodegenerative disorders (Kinger *et al*. 2024). In order to recognize and degrade proteins, the UPS use a tagging system through the action of three well-known enzymes called the ubiquitin-modifying enzymes: E1, E2 and E3 (Amm, Sommer and Wolf 2014). For UPS activation, E1 ubiquitin-activating enzyme (UBA1/UBE1) firstly interacts with ubiquitin proteins. Subsequently, the second enzyme from cascade, E2 ubiquitin conjugating enzyme, binds and activates ubiquitin and aids in its translocation to the final enzyme in cascade, E3 ubiquitin-ligase (Hershko and Ciechanover 1998; Ciechanover and Brundin 2003; Stewart *et al*. 2016).

In AD, tau filaments can impair and decrease proteasome activity, leading to neurodegeneration (Keck *et al*. 2003). Moreover, Aβ was observed to increase the E1 enzyme, probably to maintain ubiquitin-dependent proteolytic pathway (Lopez Salon *et al*. 2003; Kinger *et al*. 2024). Additionally, in neuronal cells exposed to Aβ, there was an observed induction of the ubiquitin-conjugating

enzyme E2-25 kDa/Huntingtin interacting protein 2 (E2-25K/HIP2). This increase in HIP2 was potentially linked to a compromised proteasome function (Song *et al*. 2003). Furthermore, E3 is able to degrade APP through HRD1 domain (Kaneko *et al*. 2010). However, increasing levels of insoluble HRD1 due to oxidative stress, may lead to APP accumulation and following Aβ production (Saito *et al*. 2010; Kaneko *et al*. 2012; Saito *et al*. 2014; Kinger *et al*. 2024). Unfortunately, the UPS is not the only altered network. Indeed, ALP results to be impacted as well. Under physiological conditions, Aβ undergoes lysosomal degradation, but this process appears to be impaired in AD. In this context, Pickford and coworkers demonstrated that decreased levels of the autophagic component Beclin 1 were linked to neurodegeneration, especially during the early stages of the disease (Pickford *et al*. 2008). In addition, their studies have shown that transcription factor levels, as the nuclear factor of activated T-cells (NFAT), could modulate chaperone-mediated autophagy. Indeed, NFAT is a transcription factor for lysosome-associated membrane protein 2 (LAMP2) gene (Valdor *et al*. 2014). Decreased NFAT has been linked to reduced chaperone-mediated autophagy and increased regulator of calcineurin 1 (RCAN1), which is also observed in AD. Besides transcription factors, other proteins have an important impact on macro autophagy, such as PSEN1. PSEN1 is fundamental for targeting vacuolar H^+-ATPases (v-ATPase) to lysosomes, lowering lysosomal lumen pH, essential for autophagic protein breakdown. Alterations in lysosomal proteolysis, results in toxic protein build-up and neuron death in AD (Lee *et al*. 2010; Kinger *et al*. 2024).

3. The Amyloid-β peptide

3.1 Structure and function of APP

APP is a transmembrane protein expressed in various tissues, especially in the central nervous system (CNS), where the major isoform encompasses 695 amino acid residues (Jakob-Roetne and Jacobsen 2009; Nalivaeva and Turner 2013). APP can undergo two major proteolytic pathways, the non-amyloidogenic and the amyloidogenic one (Figure 4). The non-amyloidogenic pathway begins with α-secretase-mediated cleavage of APP at amino acid 687 (in the APP 770 isoform) which releases the ectodomain, soluble APP α (APPsα), into the extracellular space. As a result, a C-terminal fragment of APP (83 amino acids in length, CTF83 or αCTF) remains embedded in the plasma membrane. Cleavage of CTF83 by γ-secretase releases a small p3 fragment into the extracellular space and the APP intracellular domain (AICD) into the cytoplasm (Nalivaeva and Turner 2013).

Unlike sAPPβ and Aβ, sAPPα was shown to possess neurotrophic and neuroprotective functions. Indeed, sAPPα reduces tau hyper-phosphorylation by inhibiting β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and glycogen synthase kinase (GSK)-3β activity in cultured cells and transgenic

presenilin 1 and APP (PSAPP) mouse model (De Strooper 2003; Obregon *et al*. 2012; Deng *et al*. 2015). The amyloidogenic processing begins with β-secretase-mediated APP cleavage at amino acid 671 (in the APP 770 isoform) and results in the release of a small and soluble ectodomain, APP β (sAPPβ), into the extracellular space. A larger APP C-terminal fragment composed of 99 amino acids (CTF99, or βCTF) remains embedded in the plasma membrane. Finally, the cleavage of CTF99 by γ-secretase releases Aβ peptide into the extracellular space and the AICD into the cytoplasm, respectively (Figure 4; Wilkins and Swerdlow 2017). Aβ peptide is a 38- to 43-amino acids residue peptide and is mainly produced intracellularly in vesicles like endosomes, and then released in the extracellular space of healthy brain during neuronal activity (Jarrett, Berger and Lansbury 1993; Saido and Leissring 2012; Cheignon *et al*. 2018).

Overall, the function of APP is not fully understood. It has been assumed to relate to several processes, such as cell adhesion, due to its interaction with laminin and collagen and its co-localization with integrins, specifically within neuronal axons (Yamazaki, Koo and Selkoe 1997; Young-Pearse *et al*. 2008). Furthermore, sAPPα may stimulate the differentiation of neuronal stem cells into an astrocytic phenotype (Caillé *et al*. 2004). sAPPβ has been described as a less potent protectant, or even as a harmful agent for neurons (Chasseigneaux and Allinquant 2012), as it can cause neuronal death and axonal pruning by binding to the death receptor 6 (DR6; Nikolaev *et al*. 2009).

3.2 Aβ peptide aggregation

Approximately 50 human pathologies are associated with the deposition of amyloid, primarily composed of a single misfolded peptide or protein (Chiti and Dobson 2017). These conditions, collectively termed protein misfolding diseases (or protein conformational diseases), arise from the conversion of soluble, functional peptides or proteins into highly ordered, insoluble fibrillar aggregates (Chiti and Dobson 2006). This aberrant aggregation process disrupts cellular homeostasis and contributes to the pathogenesis of various debilitating diseases. Among these pathologies, neurodegenerative disorders, including AD, represent nowadays very common and detrimental medical conditions in the industrialized countries. Aβ plays a main role in the process of fibrillogenesis in AD. Precisely, Aβ belongs to the class of "natively disordered" proteins, existing in the monomeric state in an equilibrium mixture of many conformers (Roychaudhuri *et al*. 2009). Moreover, its two isoforms, $A\beta_{40}$ and $A\beta_{42}$, are normal constituents of cerebrospinal fluid (CSF) (Frost *et al*. 2003). Both the $A\beta_{40}$ and $A\beta_{42}$ monomers are dominated by random coil segments in their typical ensembles (Vivekanandan *et al*. 2011; Somavarapu and Kepp 2015).

The process of fibrillogenesis begins with amyloid monomers that, through a slow and often rate-limiting process called primary nucleation (k_n), assemble to form a variety of oligomeric species. These oligomers can then undergo further aggregation into short, flexible, irregular protofibrils, or alternatively, they can

participate in secondary nucleation events (k_2), catalyzing the formation of new aggregates on their surfaces. These protofibrils mature and elongate (k_p) into insoluble fibrils, which in turn can also serve as templates for secondary nucleation, significantly accelerating the overall fibrillization process and leading to their rapid accumulation extracellularly. (Figure 5; Westermark 2005; Cohen *et al*. 2013; Chiti and Dobson 2017; Reiss *et al*. 2018). Precisely, $A\beta_{42}$ tends to accumulate in extracellular fibrils, whereas $A\beta_{40}$, which represents the large majority of produced Aβ (LaFerla, Green and Oddo 2007), tends to be more stable in the monomeric conformation (Hubin *et al*. 2014).

Aβ fibrils, whether obtained *in vivo* from AD patients or *in vitro*, consistently exhibit a threadlike morphology with diameters ranging from 7 to 13 nm, as visualized by electron microscopy (EM) or atomic force microscopy (AFM). These fibrils, often microns in length, typically comprise 2 to 8 protofilaments, each with a diameter of approximately 2 to 7 nm. These protofilaments intertwine, forming twisted structures or associate laterally to create flat ribbons. Notably, while less common, fibrils composed of a single protofilament have also been observed (Paravastu *et al*. 2008; Wasmer *et al*. 2008; Chiti and Dobson 2017). Fibrils possess a cross-β structure, in which β-strands are oriented perpendicularly to the fibril axis and assembled into β-sheets that run the length of the fibril, as initially detected using X-ray fiber diffraction (Sunde and Blake 1997; Chiti and Dobson 2017), with recent support from Fourier transform infrared spectroscopy (FTIR), solid-state nuclear magnetic resonance (ssNMR), and X-ray crystallography (Paravastu *et al*. 2008; Zandomeneghi *et al*. 2004; Eisenberg and Jucker 2012; Chiti and Dobson 2017) and Cryo-EM. Aβ fibril's structure representation showed in Figure 6 (Kollmer *et al*. 2019).

Although amyloid fibrils represent the main pathological hallmark of AD, increasing evidence suggests that small, soluble oligomers, are the major agents responsible for neurotoxicity in AD (Kayed *et al*. 2003; Benilova, Karran and De Strooper 2012; Chiti and Dobson 2017; Selkoe 2019). The size of Aβ oligomers is distributed over a wide molecular weight range (from < 10 kDa to > 100 kDa), with structural polymorphisms in oligomers of similar sizes (Sakono and Zako 2010). Indeed, Aβ oligomers are organized in different structures ranging from dimers (Sakono and Zako 2010), trimers (Sakono and Zako 2010), tetramers (Walsh *et al*. 2000, Chen and Glabe 2006), pentamers (Ahmed *et al*. 2010), nonamers and dodecamers (referred to as Aβ*56) (Sakono and Zako 2010), and amyloid derived-diffusible ligands (ADDLs; Lambert *et al*. 1998; Sakono and Zako 2010). Furthermore, previous studies have shown that oligomer size distribution was very different between $A\beta_{42}$ and $A\beta_{40}$, indicating that their oligomerization pathways are different (Bitan *et al*. 2003; Sakono and Zako 2010).

Despite the small structural difference between these two peptides, they display distinct clinical, biological, and biophysical behaviors (Bitan *et al*. 2003). The concentration of secreted $A\beta_{42}$ is ~ 10% that of $A\beta_{40}$, yet the longer form is the predominant component in parenchymal plaques (Bitan *et al*. 2003). Indeed, an increase in the $A\beta_{42}/A\beta_{40}$ concentration ratio is associated with familial forms of

early-onset AD (Scheuner *et al*. 1996; Golde, Eckman and Younkin 2000). In addition, $A\beta_{42}$ displays enhanced neurotoxicity with respect to $A\beta_{40}$ (Younkin 1995; Selkoe 1999; Dahlgren *et al*. 2002; Bitan *et al*. 2003), because of the additional presence of hydrophobic residues at the C-terminal, that makes $A\beta_{42}$ more aggregation-prone than the $A\beta_{40}$ form, and so more toxic to neurons (Finder *et al*. 2010). Lambert *et al*. reported the formation of small Aβ globular oligomers (5 nm in diameter) in Ham's-F12 medium from a synthetic $A\beta_{42}$ amyloid peptide, which were referred to as ADDLs (Lambert *et al*. 1998). Importantly, ADDLs strongly bound to the dendritic arbors of cultured neurons, blocked long term potentiation (LTP) and caused neuronal cell death (Lambert *et al*. 1998; Wang *et al*. 2002). The finding of ADDLs in soluble brain extracts from human AD brains using ADDL-specific antibodies supports the idea that their presence in human AD brain is strongly associated with the disease (Sakono and Zako 2010). Moreover, a study by Yamamoto and collaborators showed the formation of toxic Aβ oligomers in the presence of the GM1 ganglioside (Yamamoto *et al*. 2007). These oligomers were spherical, with a diameter of 10–20 nm and a molecular mass of 200–300 kDa, therefore much larger than ADDLs (Sakono and Zako 2010). Furthermore, Aβ monomers produced extracellularly can interact with GM1, and Aβ complexes with GM1 have been found in AD brains (Yanagisawa 2007). These observations support the idea that extracellular soluble Aβ oligomers could be formed by GM1 (Sakono and Zako 2010). Later, Ladiwala and coworkers characterized two $A\beta_{42}$ oligomers, referred to as A+ and A-, found that they were both globular in shape and had size of 6.2 ± 0.5 nm or 6.1 ± 0.6 nm, respectively, in height (Ladiwala *et al*. 2012). Such oligomers were also compared to ADDLs and fibrillar conformers formed at high concentrations of $A\beta_{42}$ monomeric protein, which appeared to possess an elongated morphology as observed by AFM (Figure 7) (Banchelli *et al*. 2020).

Despite their structural similarity, A- oligomers showed a much lower toxicity than the A+ oligomers when the metabolic activity was assessed through the (3-[4,5- dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reduction inhibition assay and membrane integrity was evaluated via lactate dehydrogenase (LDH) activity assay in differentiated PC12 cells and primary cultures of rat embryonic cortical neurons (Ladiwala *et al*. 2012). Furthermore, fibrils were reported to be mildly toxic to neuronal cells (Ladiwala *et al*. 2012), whereas A+ oligomers show a higher neurotoxicity due to the presence of an array of aminoacidic residues on their surface exposing hydrophobic groups, as well as tyrosine (Tyr) and lysine (Lys) residues, that are linked to a higher neurotoxicity with respect to that of A- oligomers and amyloid fibril. The detrimental effects appear to result from an aberrant interaction with cellular components, such as membrane, small metabolites, proteins or other macromolecules (Chiti and Dobson 2006; Banchelli *et al*. 2020). Schematic representation of A+ and A- oligomers is reported in Figure 8.

4. The amyloid hypothesis and pathogenic pathways

A dominant theory for the pathology of AD is the "amyloid hypothesis", which describes a complex sequence of pathogenic events responsible for neurodegeneration (Figure 9; Hardy and Allsop 1991; Selkoe 1991; Hardy and Selkoe 2002). According to this hypothesis, the aberrant accumulation of the Aβ peptide, following the amyloidogenic processing of the APP, results in the production of cytotoxic complexes, such as small and soluble oligomers, which accumulation triggers a cascade of pathogenic events, including the alteration of mitochondrial functions, oxidative stress and calcium dyshomeostasis (Braak and Braak 1994).

Although our primary focus will be on the amyloid cascade hypothesis and the description of related pathogenic pathways, it is important to acknowledge the existence and growing importance of alternative hypotheses and mechanisms implicated in the pathogenesis of AD. These include, but are not limited to, the vascular hypothesis, which emphasises the role of cerebrovascular dysfunction (Iadecola 2017), prion-like tau protein aggregation (Goedert, Masuda-Suzukake, Falcon 2017), neuroinflammation (Heneka *et al*. 2015) and metabolic dysfunction (De la Monte and Tong 2014).

The vascular hypothesis of AD posits that cerebrovascular dysfunction is a key contributor to its pathogenesis. Indeed, AD brains have microvascular alteration and more atherosclerosis in intracranial vessels than age matched controls (Love and Miners 2016; Iadecola 2017). Furthermore, it was observed that AD pathology and ischemic lesions frequently coexist in the same brain (Schneider *et al*. 2007; Toledo *et al*. 2013; Iadecola 2017), and that ischemic lesions lower the threshold for clinical dementia leading to neurodegeneration (Kapasi, DeCarli and Schneider 2017; Iadecola 2017). Beyond amyloid, the prion-like propagation of tau protein, independent of or synergistic with amyloid, is gaining support. This mechanism suggests that misfolded tau seeds can spread throughout the brain, templating further misfolding and driving neurofibrillary tangle formation (Goedert, Masuda-Suzukake and Falcon 2017). Moreover, neuroinflammation, once considered a secondary response to amyloid plaques, is now understood as a powerful driver of neurodegeneration. Indeed, in AD pathology, microglia's ability to phagocytose Aβ decreases (Hansen, Hanson and Sheng 2018) and actively contribute to neurodegeneration through the release of toxic substances and regulation of synaptic function (Hong *et al*. 2016; Miao *et al*. 2023). Furthermore, metabolic dysfunction, including insulin resistance and mitochondrial abnormalities, has been implicated in AD. These metabolic impairments can disrupt neuronal function, increase oxidative stress, and promote the accumulation of toxic protein aggregates (De la Monte and Tong 2014). It is likely that these diverse pathways interact and influence each other, contributing to the complex and heterogeneous nature of AD.

4.1 Plasma membrane disruption

The cell membrane consists of a semi permeable lipid bilayer. It regulates the transport of materials entering and exiting the cell. In 1972, Singer and Nicolson proposed the *fluid- mosaic model*, describing the cellular membrane as a double layer of lipids in a lamellar liquid-crystalline phase (Singer and Nicolson 1972). Nowadays, membranes are considered to be increasingly complex, characterized by lipid/proteins assemblies, with lateral and transverse asymmetries, different patchiness and variable thickness with an elevated protein presence (Engelman 2005; Nicolson 2014; Fabiani and Antollini 2019).

Several fundamental biochemical reactions crucial for cell life, such as metabolic and signaling reactions involving G-protein, histamine, Gamma-Aminobutyric acid (GABA) or glutamate receptors and other, take place in cell membranes, making them a key agent in almost all cellular physiological and pathological processes (Li G. *et al*. 2009; Fabiani and Antollini 2019). Lipids, such as fatty acids, cholesterol (chol), endocannabinoids, arachidonic acid metabolites such as prostaglandins, leukotrienes and epoxyeicosatrienoic acids, and many others, are major plasma membrane constituent with important implications (Fabiani and Antollini 2019). Chol, cardiolipin, phosphatidylinositol-4,5-biphosphate (PIP2) and glycolipids influence the action of numerous transmembrane proteins, as reported in several research papers (Lee 2003; Barenholz 2004; Barrera, Zhou and Robinson 2013; Fabiani and Antollini 2019).

In AD, Mason and coworkers in 1992 demonstrated that isolated brain membranes derived from AD patients had lower levels of cholesterol, disadvantaging the insertion of Aβ into the membrane (Mason *et al*. 1992). Thus, Aβ remains attached to the cellular membrane surface leading to plaques formation (Ji, Wu and Sui 2002). Taking advantage of confocal microscopy and fluorescence anisotropy, Cecchi and colleagues showed an inverse correlation between chol content and membrane perturbation (Cecchi *et al*. 2009). It has also been shown that increasing chol reduces amyloid-induced membrane disruption by modifying the raft domain properties (Cecchi *et al*. 2009; Fabiani and Antollini 2019).

Another important lipid in Aβ-membrane interaction is GM1, an important component of the raft domains (Lin *et al*. 2008), as previously reported. Precisely, increasing chol levels enhance gangliosides clustering, which is supposed to influence and modulate Aβ oligomerization. The interaction between clusters and Aβ peptides can induce Aβ aggregation in β-sheet rich structures in a concentration dependent way (McLaurin *et al*. 1998; Ariga *et al*. 2001; Kakio *et al*. 2001, 2002; Matsuzaki 2007; Fabiani and Antollini 2019). The binding Aβ/GM1 cluster promotes a conformational change depending on the protein density of the membrane. At low peptide/lipid ratios the transition from random coil to α-helix conformation is favored, while at high peptide/lipid ratios the fibrils formation is promoted, due to Aβ-sheet rich structure organization (Matsuzaki, Kato and Yanagisawa 2010; Fukunaga *et al*. 2012; Cascella *et al*. 2017; Fabiani

and Antollini 2019). Representative interaction Aβ/ganglioside is shown in Figure 10. Closer examination of the mechanism of Aβ interaction with GM1 suggests that Aβ oligomers, which have increased hydrophobicity compared to Aβ monomers as reported in *section 3.2*, mainly bind to GM1, activating sequential changes such as membrane biophysical and ion permeability disruption, leading to Aβ-mediated synaptotoxicity (Hong *et al*. 2014; Fabiani and Antollini 2019).

Notwithstanding, all the evidence leads us to assess a direct role of gangliosides in Aβ oligomerization, another Aβ oligomerization mechanism has also been observed, without being dependent on Aβ/ganglioside interaction, suggesting that other lipid components or raft glycans may also be involved (Kim, Yi and Ko 2006; Fabiani and Antollini 2019).

In addition to the role of lipids in Aβ-induced neurotoxicity, three main membrane damage models have been proposed (Cheng *et al*. 2013).

The first model is based on the generation of a stable transmembrane protein pores determined by the interaction between amyloidogenic protein, in its monomeric or oligomeric form, with the membrane lipid bilayer (Sciacca, La Rosa and Milardi 2021). Consistently, Pollard and colleagues reported that Aβ interacting with lipid membranes could lead to the formation of calcium (Ca^{2+})-permeable channels, inducing cell death (Pollard, Rojas and Arispe 1993; Sciacca, La Rosa and Milardi 2021). Indeed, other evidence suggested that blocking Ca^{2+} influx reduces the Aβ oligomer neurotoxicity and insoluble $Aβ_{40}$ and $Aβ_{42}$ levels in AD mice (Samad *et al*. 2017; Cascella and Cecchi 2021). Moreover, elevated intracellular Ca^{2+} activates calcineurin (CaN) and phosphatases like PP1, involved in long-term depression (Reese and Taglialatela 2011; Cascella and Cecchi 2021).

The second model is represented by the membrane destabilization via a "carpet model", determined by the interaction of prefibrillar species with the cellular membranes resulting in an asymmetric pressure between both layers (Sciacca, La Rosa and Milardi 2021). Proximal or distant pressure relief with respect to the protein determines leakage of small molecules, leading to membrane disruption (Hebda and Miranker 2009; Sciacca, La Rosa and Milardi 2021).

The third model is described as a "detergent-like mechanism". This model is determined by an asymmetric pressure caused by peptides carpeting the cellular membrane, leading to the loss of lipid from one or both leaflets of the bilayer (Bode *et al*. 2019; Sciacca, La Rosa and Milardi 2021). Precisely, this detergent-like mechanism may result in a thinner membrane or in the formation of a hole (Sciacca, La Rosa and Milardi 2021).

In conclusion, the Aβ-induced neurotoxicity described through these three mechanisms contributes to the neuronal damage observed in AD. Indeed, a deep understanding of these mechanisms is crucial for the development of therapeutic strategies counteract disease progression. A schematic representation of the aforementioned models in Figure 11.

4.2 Mitochondrial dysfunction

Mitochondrial dysfunction plays a crucial role in the pathogenesis of AD (Rai *et al*. 2020). Mitochondria are ubiquitous intracellular organelles and they represent the primary source for the generation of biological energy in the cell in the form of ATP (Cadonic, Sabbir and Albensi 2016; Bhatia *et al*. 2022). Mitochondria contain an outer membrane, which is freely permeable to small molecules and ions, and an inner one, impermeable to most molecules and ions (Cadonic, Sabbir and Albensi 2016), which contains the electron transport chain (ETC), ADP-ATP translocases, ATP synthases, and numerous other membrane transport systems. The ETC and the ATP synthases dispersed throughout this membrane are directly involved in the generation of ATP from the potential energy stored in substrates (*i.e*. glucose) (Cadonic, Sabbir and Albensi 2016). In the early 1980s, fluorodeoxyglucose positron emission tomography (FDG PET) studies showed that brains of AD patients utilized less glucose than those of control subjects, thus leading to confusion and loss of temporary memory in the early stages of disease (Swerdlow 2018). Further evidence of impaired glucose metabolism in AD patients comes from the cohort autopsy conducted within the Baltimore Longitudinal study of aging evaluating the ratios of concentrations of glycolytic amino acids, serine, glycine, and alanine to glucose and quantifying the protein levels of neuronal (GLUT3) and astrocytic (GLUT1) transporters (An *et al*. 2018). The study revealed that the severity of AD symptoms were directly linked to the decreased glycolytic flux that downregulate GLUT3 levels and increase tissue glucose concentrations, establishing that impaired glucose metabolism was connected to the progression of AD (An *et al*. 2018; Bhatia *et al*. 2022). In addition, Aβ can enter the matrix through the TOM/TIM channels, thus leading to mitochondrial dysfunction (Hashimoto *et al*. 2003). Indeed, cultured PC12 cells maintained in the presence of $A\beta_{42}$ oligomers showed reduced electron transport chain enzyme activities (Pereira, Santos and Oliveira 1998; Swerdlow 2018). Post-mortem studies on AD patients have revealed a decrease in the levels and activity of α-ketoglutarate dehydrogenase complex (a rate-limiting enzyme of the TCA cycle) in the temporal cortex, parietal cortex, and hippocampus regions (Sheu *et al*. 1994; Mastrogiacoma *et al*. 1996; Kish 1997; Bhatia *et al*. 2022). Cytochrome oxidase and pyruvate dehydrogenase enzymes were also found to be deficient in AD patients (Sorbi, Bird and Blass 1983; Sheu *et al*. 1985; Parker, Filley and Parks 1990, Bhatia *et al*. 2022). $A\beta_{42}$ oligomers were also reported to impair respiratory chain function in isolated mitochondria (Canevari, Clark and Bates 1999; Swerdlow 2018). Moreover, Aβ can affect mitochondrial function by binding heme groups, which reduces their functionality as redox centers in the ETC. This event occurs predominantly at the heme groups of complex IV of the ETC (Santos *et al*. 2010; Cadonic, Sabbir and Albensi 2016). Furthermore, Aβ may also bind to Aβ-binding alcohol dehydrogenase (ABAD), resulting in an increase in reactive oxygen species (ROS) generation through impairment in complex IV molecular O2 processing, reduced cytochrome c release, and decrease in ATP production (Santos *et al*. 2010; Cadonic, Sabbir and

Albensi 2016). In the early stages of AD, a reduced axonal transport of mitochondria has been reported, as well as fewer mitochondria within neurons, with the remaining organelles presenting significant alterations in size and shape (Esteves and Arduino 2009; Zhu *et al*. 2013). Mitochondrial dysfunctions reported above are represented in Figure 12.

4.3 Calcium dyshomeostasis

Calcium ions (Ca^{2+}) regulate the function of various enzymes and proteins and play an important role as secondary messengers in signal transduction pathways, including cell survival, proliferation, differentiation and apoptosis (Clapham 2007; Cascella and Cecchi 2021). Ca^{2+} is also involved in the regulation of multiple neuronal and astrocytic functions, such as neurotransmitter release, synaptic plasticity, membrane excitability, gene transcription, proliferation and cell death (Berridge 1998; Berridge, Lipp and Bootman 2000; Cascella and Cecchi 2021). A wide variety of Ca^{2+} channels are present in the plasma membrane with a diverse distribution. In particular, N-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) regulate the Ca^{2+} influx from extracellular to intracellular space by performing fine-tuning (Cascella and Cecchi 2021).

The "calcium hypothesis", firstly postulated by Khachaturian in 1989 following major experimental studies by the group of Landfield and colleagues, investigated how the activation of the amyloidogenic pathway can reshape neuronal Ca^{2+} signaling pathways responsible for cognition (Khachaturian 1989). According to this hypothesis, the depolarization-repolarization cycles in which Ca^{2+} influx from the extracellular space plays a critical role, are responsible for many of the neuronal dysfunction observed in brain disorders in older people. Several studies support the notion that embrane disruption induced by Aβ aggregates allows the entry of small molecules and ions, mainly free Ca^{2+} (Peters *et al*. 2015). Moreover, it was reported that Aβ cause the formation of stable pores and ion channels, usually defined as "annular protofibrils", thereby resulting in perturbation of Ca^{2+} influx (Lin, Bhatia and Lal 2001; Kagan, Azimov and Azimova 2004; Arispe, Diaz and Simakova 2007). Based on these findings, numerous researchers have focused their attention on the effects of Aβ aggregates on Ca^{2+} channels in neuronal cells. Specifically, the blockade of Ca^{2+} influx was reported to reduce the neurotoxicity of Aβ oligomers and the levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus of AD transgenic mice (Samad *et al*. 2017; Cascella and Cecchi 2021). Increasing evidence suggests that one of the most important Aβ targets is NMDAR (Popugaeva, Pchitskaya and Bezprozvanny 2018). NMDARs are cationic channels gated by the neurotransmitter glutamate, having critical roles in excitatory synaptic transmission, plasticity, as well as in excitotoxicity in the CNS (Kamat *et al*. 2016); their activation leads to massive Ca^{2+} fluxes into the postsynaptic cells (Kamat *et al*. 2016). Previous studies have shown that Aβ in its oligomeric form can increase the vulnerability of neurons to excitotoxicity,

which is caused by excessive NMDARs activation with subsequent cell calcium overload (Mattson *et al*. 1992; Mattson 2004; Popugaeva, Pchitskaya and Bezprozvanny 2018). The resulting activation of downstream signal transduction pathways triggers a cascade of pathological events leading to synaptic disruption and neuronal loss. In addition, Aβ-induced synaptic degeneration also involves surface removal and endocytosis of AMPARs, thus increasing calcium dysregulation and neuronal loss (Tu *et al*. 2014). Neurotoxic mechanisms induced by Aβ oligomers are briefly reported in Figure 13.

4.4 Oxidative stress

Oxidative stress is a condition produced by the imbalance between oxidants and antioxidants in a biological system (Singh *et al*. 2019). Neuronal vulnerability to ROS is emerging as a key detrimental factor responsible for AD pathogenesis. The brain consumes a large amount of oxygen for proper functioning, and mitochondria present in neuronal cells are responsible for the production of a large amount of free radicals and ROS, due to the high accessibility of oxygen in these organelles (Angelova and Abramov 2018).

In pathogenic conditions, such AD, dysfunctional mitochondria are less efficient producers of ATP but more efficient producers of ROS (Wang *et al*. 2014). ROS significantly contribute to the deterioration of neuronal cells via modulating the function of biomolecules (DNA, RNA, lipids, and proteins), and processes (nucleic acid oxidation, lipid peroxidation) in the cell (Halliwell and Gutteridge 2015). The ROS primarily involved in neurodegeneration are hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), and the highly reactive hydroxyl radical (HO^-). In addition, reactive nitrogen species (RNS), such as nitric oxide (NO) also have a deleterious effect on neurons (Singh *et al*. 2019). Several studies have shown that Aβ-induced oxidative imbalance is responsible for the elevated levels of lipid peroxidation by products (*e.g*., 4-hydroxynones, malondialdehyde), protein oxidation (*e.g*., carbonyl), and DNA/RNA oxidation (*e.g*., 8-hydroxylguanosine, 8-hydroxydeoxyguanosine) (Praticò 2008; Zhao and Zhao 2013; Wang *et al*. 2014; Singh *et al*. 2019).

Moreover, increasing evidence has shown that various metals are involved in the pathogenesis of AD, such as iron, zinc, copper. Many enzymes require metals for their proper function, but the interaction of Aβ with redox active metals hinders their enzymatic function. Furthermore, the binding of Aβ to copper in its redox state promotes the aggregation process (Greenough, Camakaris and Bush 2013; Singh *et al*. 2019). Finally, zinc dyshomeostasis occurs in the inflammatory response to insoluble Aβ plaques, leading to abnormal zinc release and increased oxidative stress (Huang, Zhang and Chen 2016; Singh *et al*. 2019).

5. Biomarkers

The term "biomarker" or "biological marker" refers to a broad subcategory of medical signs that can be measured accurately and reproducibly (Strimbu and

Tavel 2010). In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as an indicator of a normal, or either pathogenic, biological process, or a pharmacological response to a therapeutic intervention that can be evaluated and measured (Biomarkers Definitions Working Group 2001; Strimbu and Tavel 2010).

5.1 The A/T/N classification

The A/T/N classification system for AD biomarkers, where "A" indicates Aβ biomarker concentrations, "T" identifies the level of tau biomarkers, and "N" refers to neurodegeneration biomarkers, is used to distinguish three groups of biomarkers based on different neuropathological mechanisms (Jack *et al*. 2018).

The first category involves indicators of Aβ deposition: the CSF of AD patients is characterized by a reduced level of $A\beta_{42}$ concentration (by ~50% with respect to healthy subjects) (Olsson *et al*. 2016; Zetterberg and Bendlin 2021). $A\beta_{42}$ is normally mobilized from the interstitial fluid of the brain into the CSF and blood, probably through the lymphatic system (Rasmussen, Mestre and Nedergaard 2018). The reduction in $A\beta_{42}$ levels in AD is due to its aggregation in the brain parenchyma. Diagnostic accuracy for Aβ pathology can be increased by dividing the aggregation-prone $A\beta_{42}$ concentration by soluble $A\beta_{40}$; both are products of the same APP-processing pathway, but $A\beta_{40}$, in contrast to $A\beta_{42}$, remains soluble in AD. The CSF $A\beta_{42}/A\beta_{40}$ ratio is influenced by interindividual differences in amyloidogenic APP-processing (high vs. low Aβ producers) and is close to 100% concordant with amyloid PET (Hansson *et al*. 2019; Zetterberg and Bendlin 2021); discordant cases CSF-positive and PET-negative, often become PET-positive within a few years (Lewczuk *et al*. 2017; Hansson *et al*. 2019; Zetterberg and Bendlin 2021).

The second major AD neurodegenerative biomarkers are increased levels of CSF total (t-tau) and phosphorylated (p-tau) tau protein (Fagan *et al*. 2009; Shaw *et al*. 2009; Jack and Holtzman 2013), atrophy on structural MRI (Vemuri *et al*. 2009; Dickerson and Wolk 2012; Jack and Holtzman 2013), and hypometabolism on FDGPET (Jagust 2010). Blennow and coworkers demonstrated that tau pathology could be reflected with biomarkers through ELISA assay capturing specifically tau protein phosphorylated threonine 181 (p-tau 181) and 231 (p-tau231) (Blennow *et al*. 1995). P-tau species seem to predict progression from MCI to dementia (Mattsson *et al*. 2009) and are highly accurate in differential diagnosis of AD (Ewers *et al*. 2015). In accordance with the National Institute on Aging and the Alzheimer's Association (NIA-AA) research framework and novel International World Group (IWG) criteria, another biomarker proposed to reflect tau pathology is PET imaging, targeting AD-specific tau deposits, which do not occur in other neurodegenerative diseases with tau pathology (Ossenkoppele *et al*. 2018; Simrén *et al*. 2023).

The third major category is neurodegeneration, defined as the process of progressive neuronal loss. Importantly, CSF levels of $A\beta_{42}$ and amyloid PET are

specific for Aβ deposition, whereas these changes are not specific in AD (Jack *et al*. 2002; Simrén *et al*. 2023). In subjects with AD, both FDG PET and MRI follow a modality-specific pattern that is typical of AD (Senjem *et al*. 2005; Jack and Holtzman 2013). One of the first pathological alterations is the temporal lobes atrophy mainly in the hippocampal region. Although it is not a specific diagnostic marker, rate of change predicts disease progression accurately (Frisoni *et al*. 2010). Generally, an MRI is highly recommended for patients seeking medical advice for cognitive decline (Simrén *et al*. 2023). Complementary, FDG PET gives information on decreased neuronal glucose metabolism. This technique not only assesses functional decline but also directly measures neuronal loss, typically observed in the posterior cingulate and temporo-parietal regions. The distinct patterns associated with various neurodegenerative diseases make this a valuable tool for differentiating between them (Chételat *et al*. 2020; Simrén *et al*. 2023). Fluid biomarkers proposed to reflect neurodegeneration involve CSF neurofilament light (NfL), the main component of the neuroaxonal cytoskeleton (Friede and Samorajski 1970). Unfortunately, the CSF sampling is an invasive and expensive procedure and amyloid PET has limited availability (and not insignificant radiation exposure); consequently, a blood biomarker for brain Aβ pathology would be an important step toward precision medicine and to perform clinical follow-up of patients with cognitive disorders (Olsson *et al*. 2016; Zetterberg and Bendlin 2021; Mielke and Fowler 2024). Recently, it has been observed that $A\beta_{42}$ levels in relation to $A\beta_{40}$ (measured by immunoprecipitation mass spectrometry or ultrasensitive enzyme-linked immunosorbent assays) reflect brain Aβ pathology with relatively high accuracy as compared to both amyloid PET and CSF $A\beta_{42}/A\beta_{40}$ ratio (Janelidze *et al*. 2016; Ovod *et al*. 2017; Zetterberg and Bendlin 2021), although evaluation against neuropathology is equally necessary (Zetterberg and Bendlin 2021). The main synaptic biomarkers are neurogranin (Ng) in CSF, as well as the synaptic vesicle glucoprotein 2A (SV2A). The most established astrocytic biomarker is the chitinase-3-like protein 1 (YKL-40) in CSF, and another promising biomarker is glial fibrillary acidic protein (GFAP) in CSF and plasma that highlights astrocytic activation/degeneration (Antonell *et al*. 2014; Zetterberg and Bendlin 2021). An overview of the AD biomarkers continuum is reported in Table 1.

Recently, new criteria for AD diagnosis have been introduced, expanding the biological framework for AD diagnosis and specifying further categories within the ATN system. In this context, *α*-Synuclein (*α*Syn) was found to be involved in the pathophysiology of AD (Twohig and Nielsen 2019). In 2024, Bellomo and coworkers found a significant proportion of AD patients (30%) being positive to *α*Syn seed amplification assays (*α*S-SAAs) in all clinical stages of AD. Furthermore, the *α*S-SAAs positivity was associated with a more marked cognitive decline (Bellomo *et al.* 2024). From this evidence, the introduction of synucleinopathy as a new criterion for AD diagnosis represent a successful strategy for the selection of AD patients who may benefit from anti-*α*Syn therapies (Bellomo *et al*. 2024).

5.2 Temporal evolution of AD biomarkers in the CSF

Biomarkers could change linearly with time, exponentially or with a sigmoidal way. Initially, the temporal evolution of AD biomarkers was based on observations in elderly subjects through the cognitive continuum (Jack *et al*. 2008, 2009, 2010a; Mormino *et al*. 2009; Perrin, Fagan and Holtzman 2009; Jack and Holtzman 2013). These initial models assumed that amyloid biomarkers were the first to become abnormal, followed by biomarkers of tau-related neurodegeneration and finally by overt clinical symptoms. Notably, a further model proposed that the general or functional form for biomarker evolution was sigmoidal with time (Jack *et al*. 2010a).

Subsequently, empirical studies in elderly individuals (Buchhave *et al*. 2012; Förster *et al*. 2012; Jack *et al*. 2010b; Landau *et al*. 2012; Lo *et al*. 2011; Villemagne *et al*. 2011, 2013; Jack and Holtzman 2013) and in autosomal dominant mutation carriers (Bateman *et al*. 2012; Reiman *et al*. 2012; Jack and Holtzman 2013) showed that the order of biomarker alteration was consistent with that proposed in models. In addition, these data also showed that the class of biomarkers related to Aβ followed a sigmoidal shape over time (Caroli and Frisoni 2010; Fleisher *et al*. 2012; Jack *et al*. 2012, 2013; Villemagne *et al*. 2013; Jack and Holtzman 2013).

In 2013, Jack, Jr. and Holtzman proposed three different sets of biomarker models, each culminating in the common clinical phenotype of AD dementia (Jack and Holtzman 2013). Specifically, they proposed the "Pure AD biomarker model: Amyloid first", where the temporal ordering pattern in this model corresponds to the sequence of molecular events proposed in the amyloid cascade hypothesis (Glenner and Wong 1984; Hardy and Selkoe 2002; Jack and Holtzman 2013). Initially, this model is characterized by the overproduction and aggregation of $A\beta_{42}$ and decreased clearance, resulting in the formation of amyloid plaques. CSF $A\beta_{42}$ and amyloid PET are the first biomarkers to become abnormal. Following the deposition of $A\beta_{42}$ aggregates, tauopathy is induced in the medial temporal lobe and promotes its spread into the neocortex. Tauopathy results in detectable abnormalities in MRI and FDG PET imaging biomarkers of structural neurodegeneration. At this stage, clinical symptoms follow the MRI and FDG PET abnormalities (Reed *et al*. 2010; Vemuri *et al*. 2011). In the early and intermediate clinically symptomatic stages of the disease, amyloid biomarkers approach a plateau while MRI, FDG PET, and tau tend to increase (Jack and Holtzman 2013). In the late phase of the disease, all biomarkers approach a plateau. Unfortunately, systematic studies on the progression of biomarkers in the end stage of disease have not been done because of difficulties in performing (Jack and Holtzman 2013). An explicative model of temporal evolution of AD biomarkers is represented in Figure 14.

5.3 Putative CSF biomarkers based on the novel concept of generic protein misfolding and proteotoxicity

The current literature demonstrates a clear association between protein misfolding and the deterioration of PN, with numerous studies indicating that disrupted PN status is linked to an elevated propensity for proteins to lose solubility and form various types of self-assemblies that are often challenging to detect (Hipp, Park and Hartl 2014). As previously mentioned in *section 2*, in the AD pathological conditions, the PN status results to be highly disrupted and unregulated, leading to the formation of misfolded oligomers and highly structured fibrils. In particular, small oligomers composed of the $_{42}$-residue form of Aβ, formed in the initial stages of the aggregation process or released from mature fibrils, have emerged as a significant focus in the study of AD pathogenesis (Kayed *et al*. 2004; Benilova, Karran and De Strooper 2012; Evangelisti *et al*. 2016; Bigi *et al*. 2023b; Bigi e*t al*. 2024b). Indeed, elevated oligomer levels in the brain have been linked to the progression of pathology and have been shown to correlate more strongly with the severity of dementia than mature fibrils (Cline *et al*. 2018; Selkoe 2019; Bigi *et al*. 2024b). Therefore, the ageing-related accumulation of protein aggregates is both a result and a cause of PN decline, creating a vicious cycle that ultimately leads to its collapse (Balchin, Hayer-Hartl and Hartl 2016; Labbadia and Morimoto 2015). Thus, the presence of aggregates of a specific protein increases the propensity of numerous others to undergo aggregation into proteotoxic oligomeric species. This scenario is evidenced by the observation that in every neurodegenerative disease the primary protein deposits are often associated with deposits of other proteins that are neither the cause nor a defining feature of that disease (Ferrer *et al*. 2001; Amador-Ortiz *et al*. 2007; Nakashima-Yasuda *et al*. 2007; Schwab *et al*. 2008; Colom-Cadena *et al*. 2013; Sengupta *et al*. 2015; Hepp *et al*. 2016; Nonaka, Masuda-Suzukake and Hasegawa 2018; Twohig and Nielsen 2019).

In this context, Bigi and Fani with their colleagues, decided to compare CSF samples derived from AD patients and control subjects in a novel Italian study named PRAMA (Proteomics, Radiomics & Machine learning-integrated strategy for precision medicine for Alzheimer's). Precisely, we investigated the presence of protein instability with biophysical methods, and correlated proteotoxicity, in the form of misfolded protein oligomers able to induce cell dysfunction in cultured cells using cell viability assays (Bigi *et al*. 2024a).

This approach expands beyond the detection of misfolded $A\beta_{42}$ and tau proteins, which constitute a minor fraction of the CSF proteome. Instead, it focuses on analysing the entire CSF proteome for misfolded proteins, grounded in the experimental evidence that AD involves a broader failure of protein homeostasis and the potential presence of misfolded protein species beyond Aβ and tau (Bigi *et al*. 2024a).

Our findings indicated that AD CSF samples exhibited elevated solvent exposure of tryptophan residues within the proteome, increased concentrations of large protein species, and elevated concentrations of protein species capable of

inducing an influx of calcium ions from the extracellular space to the cytosol of cultured cells. We can assess that these observations suggest the presence of protein misfolding, protein aggregation, and proteotoxicity. This result broadens the scope of our investigation to encompass the entire proteome, rather than focusing on specific proteins such as $A\beta_{42}$, t-tau, p-tau and numerous other individual proteins that are currently being explored as potential AD biomarkers in clinical settings (Bigi *et al*. 2024a).

6. Blood-based biomarkers in AD

The invasiveness of the lumbar puncture (LP) and the high cost of clinical procedure, such as PET imaging, make these two techniques not suitable for extensive use in primary care (Löppönen *et al*. 2003; Simrén *et al*. 2023). For this reason, researchers have felt the need to switch from CSF to blood-based biomarkers, mainly due to the large availability of samples and the drastic reduction in costs. Unfortunately, early attempts to detect and measure brain proteins in blood were extensively unsuccessful (Olsson *et al*. 2016). Finally, the development of ultrasensitive assays has made it possible to translate the CSF biomarkers reflecting AT(N) pathologies into the blood (Simrén *et al*. 2023). Although blood biomarkers are promising for early diagnosis and monitoring of AD, their diagnostic accuracy is not yet comparable to that of CSF biomarkers. Therefore, CSF analysis remains the gold standard for confirmation of diagnosis (Leuzy *et al*. 2022).

6.1 The A/T/N classification in plasma

For the first plasma biomarkers category, only recent studies have reported lower plasma $A\beta_{42}/A\beta_{40}$ in AD patients, being in accordance with PET or CSF measures. The first pioneering study was conducted by Ovod and colleagues only in 2017 (Ovod *et al*. 2017). The primary challenge with plasma Aβ as a biomarker lies in its small fold-changes between amyloid-positive and -negative individuals, potentially due to matrix effects and peripheral Aβ expression (Benedet *et al*. 2022; Simrén *et al*. 2023). This contributes to its modest association with CSF $A\beta_{42}/A\beta_{40}$ and Aβ PET, necessitating highly robust assays to minimize misclassifications arising from pre-analytical and analytical variations (Benedet *et al*. 2022). The weak correlation between current candidate methods (Pannee *et al*. 2021) further underscores the need for substantial advancements before their clinical utility can be established beyond research settings.

Concerning the second biomarkers category, recent and promising studies have shown that highly specific immunoassays for p-tau181 (Karikari *et al*. 2020), p-tau217 (Palmqvist *et al*. 2020), and p-tau231 (Ashton *et al*. 2021) are able to detect AD early changes in tau release long time before the onset of symptoms (Palmqvist *et al*. 2020; O'Connor *et al*. 2021; Simrén *et al*. 2023). Accordingly, Janelidze and coworkers have shown that plasma p-tau predicts conversion from a cognitively unimpaired condition (CU) to MCI and finally to

AD dementia (Janelidze *et al*. 2020). For p-tau, the diagnostic accuracy, as determined by the area under the curve (AUC), distinguishes symptomatic AD from differential diagnoses and healthy controls consistently around 0.9 in most studies conducted in well-characterised research cohorts. (Karikari *et al*. 2020; Palmqvist *et al*. 2020; Ashton *et al*. 2021). In conclusion, plasma p-tau might represent the most promising biomarker for use in routine clinical chemistry as a complement of CSF biomarkers, as well as for use in clinical trials as a tool for prescreening and enrichment strategies (Simrén *et al*. 2023).

For the latter plasma biomarkers category, NfL in blood strongly correlates with NfL CSF (Gisslén *et al*. 2016; Hansson *et al*. 2017; Giacomucci *et al*. 2022). However, the interindividual differences observed in seemingly healthy elderly individuals are substantial due to the presence of concomitant pathologies affecting the central and peripheral axons of the older population. Therefore, the specificity is limited. Nevertheless, a very low NfL concentration (*i.e*. clearly normal) is unlikely to be compatible with neurodegenerative diseases, such as AD (Ashton *et al*. 2021; Simrén *et al*. 2023). A further potential application of NfL is to assess the efficacy as disease- modifying drugs become more widely available. Moreover, effective treatment should result in a decline in NfL levels, as observed in individuals affected by spinal muscular atrophy (SMA), a genetic neurodegenerative disease that primarily affects children. (Olsson *et al*. 2019) In conclusion, NfL is a stable biomarker, demonstrating consistency throughout the analytical, pre-analytical and biological phases, and holds promise as a diagnostic indicator, as well as a tool for monitoring treatment efficacy and disease progression in AD. An overview of plasma biomarkers in AD is reported in Table 2.

6.2 Antibodies as diagnostic and therapeutic tools for AD

One of the greatest unmet medical challenges facing our society is the development of effective methods for diagnosing and treating of AD. Notably, the identification of early diagnostic biomarkers would enable the development of new and effective treatments for this disease (Yu and Watts 2013).

Antibodies, also known as immunoglobulins, are proteins with a high binding specificity with the target. The structure of a classical immunoglobulin G (IgG) consists of four chains, as reported in Figure 15: two heavy chains and two light ones. The light chains have a variable region (V_L) and a constant one, whereas the heavy chains possess a variable region (V_H) and a constant one, divided into three homology regions (Edelman 1970). These chains are held together by weak force and by interchain sulfur-sulfur bonds between corresponding pairs of cysteines (Edelman 1970).

The target recognition of the antigen is performed by both the variable portions of the light and heavy chains (Edelman 1970), thus making antibodies highly accurate tools for the recognition of biomarkers of pathological interest, due to their high specificity and affinity for target (Perchiacca *et al*. 2012).

Antibodies can be divided into monoclonal (mAbs) (Milstein 1980), that bind a single epitope, and polyclonal antibodies, that recognize multiple epitopes on the same antigen.

7. Core immunochemistry approaches for AD research and clinical practice

7.1 ELISA assay

Nowadays, the clinical routine measurement procedures are based on enzyme-linked immuno-sorbent assays (ELISA) (classical or automated, such as the Lumipulse ® and Elecsys® technology) (Veerabhadrappa *et al*. 2020), but there have been several approaches and attempts to detect levels of CSF biomarkers.

Major efforts have been made to develop an ELISA assay specific for Aβ oligomers, the Aβ conformer considered to be the most toxic one. Unfortunately, their detection in CSF sample derived from AD patients is very challenging given their heterogenous and transient nature and their low concentration in biological fluids. Indeed, CSF $A\beta_{42}$ oligomers concentration ranged between 0.1 and 10 pg/ml (Savage *et al*. 2014).

In 2007, it was developed an assay using flow cytometry in conjunction with fluorescence resonance energy transfer, revealing that oligomeric Aβ is detectable in control patients even without dementia (Santos *et al*. 2007). Later, a sandwich ELISA assays with a single antibody (Ban50) have been used to detect high molecular weight (HMW) oligomers demonstrating that an increase in Aβ oligomers in CSF is associated with cognitive decline (Fukumoto *et al*. 2010; Herskovits *et al*. 2013). Another measurement procedure includes the Alzbio3 assay, a multiparametric test performed on the Luminex platform. This assay is able to detect Aβ and tau proteins in the same sample and has a diagnostic accuracy similar to traditional flat plate ELISAs (Le Bastard *et al*. 2013; Herskovits *et al*. 2013).

7.2 Single Molecule Array (SIMOA)

In 2010, Rissin and coworkers introduced a new technique called Single Molecule Array (SIMOA) (Rissin et al. 2010). This assay employs an antibody-coated bead to capture the target antigen, followed by a biotinylated detection antibody. The construct is then labeled with streptavidin-β-galactosidase (SBG). Upon addition of a fluorogenic substrate, β- galactosidase activity generates fluorescent product within each well. This digital readout, where each bead represents a single molecule, enables the detection of analytes at concentrations far below the limits of conventional ELISA. The fluorescence intensity within the sample well is subsequently quantified using spectrofluorimetry. This measurement is then compared against a standard curve with known concentrations, enabling the calculation of the analyte's concentration within the sample. The primary distinction lies in the use of antibody-coated microscopic

beads in Simoa, which allows for the isolation of individual antigen-bead complexes and the application of a Poisson distribution for signal analysis, enhancing sensitivity compared to standard ELISA (Rissin et al. 2010). The principle of Simoa is represented in Figure 16.

In their study, Chatziefstathiou and coworkers, tested the diagnostic performance of a panel of four biomarkers Glial fibrillary acidic protein (GFAP), NfL, tau, Ubiquitin Carboxyl-Terminal Hydrolase L1 (UCH-L1) in CSF samples derived from AD and FTD patients and control subjects using the SIMOA platform. They demonstrated that GFAP and tau were the most valuable markers to discriminate AD from FTD patients, while NfL, tau and UCH-L1 were most valuable to differentiate between FTD and control subjects (Chatziefstathiou *et al*. 2024).

In another study, Wojdała and colleagues, evaluated two different immunoassays targeting different regions of the Aβ peptide performing a method comparison in both CSF and plasma and assessing the diagnostic performance across the AD continuum (Wojdała *et al*. 2024). They observed that while both $A\beta_{42}/A\beta_{40}$ assays allowed them to effectively discriminate between control subjects and different AD stages, the assay targeting the amyloid N-terminal region provided the best diagnostic performance in plasma (Wojdała *et al*. 2024). From both these studies, the SIMOA techniques proved to be an excellent tool, able to discriminate between different AD biomarkers and neurodegenerative diseases, assessing its differential diagnostic potential.

7.3 Chemiluminescence immune-sorbent assay (CLEIA) and electrochemiluminescence immune-sorbent assay (ECLIA).

CLEIA and ECLIA are alternative methods which adopt chemiluminescence to generate signal. Basically, chemiluminescence techniques are based on the generation of luminescence, meaning that radiation is emitted, which is being generated when an electron transits from an excited state to the ground one (Simrén *et al*. 2023). The resultant energy in the atom is released in the form of light, which is then detected (Cinquanta, Fontana and Bizzaro 2017).

The classical CLEIA is based on mixing the necessary reagents in a proper and controlled manner, whereases the ECLIA reaction is activated by changing an electrode potential. This allows the control of both the time of the reaction and the position (Miao 2008; Simrén *et al*. 2023). Nowadays, fully automated platforms used for measuring different CSF biomarkers in clinical routine are provided by Elecsys on the Cobas platform (Roche), and Lumipulse (Fujirebio) utilizing ECLIA and CLEIA, respectively (Simrén *et al*. 2023).

8. Sequence-specific and conformational antibodies against Aβ

Immunotherapy using anti-Aβ antibody is one of the most promising approaches for AD treatment (Murakami 2014). Antibodies can be divided into sequence-specific and conformational antibodies. Sequence-specific antibodies

are able to recognize six to ten amino acid residues and they are used to identify the primary structure of the protein (Edelman 1970). Indeed, the monoclonal sequence-specific antibodies 6E10 was the first anti-amyloid monoclonal antibody against Aβ and one of the most widely used antibodies in AD research. In particular, the epitopes for 6E10 maps to residues 1-16 of Aβ (Baghallab *et al*. 2018). However, most clinical studies using conventional sequence- specific antibodies have encountered significant difficulties. This is presumably due to their aspecific detection of non-pathological conformers, such as monomer, fibrils of Aβ and APP, together with pathological oligomers (Murakami 2014).

Conversely, conformation dependent antibodies have been reported to specifically recognize distinct assembly states of amyloids, such as prefibrillar oligomers or fibrils (Kayed *et al*. 2007). In 2001, Lambert and colleagues demonstrated that ADDLs are effective antigens for the induction of antibodies with therapeutic potential (Lambert *et al*. 2001). Two antibodies, referred to as M93 and M94, detected assembled forms of Aβ, which were toxic both *in vitro* and *in vivo*, but weakly associated with Aβ in its physiological monomeric state. Deepening their potency and specificity, this study demonstrated that antibodies are useful for screening compounds that delay ADDL formation and enable ADDLs detection both in cultured hippocampal neurons and in the AD affected brain (Lambert *et al*. 2001).

Two years later, Kayed and colleagues developed the A11 polyclonal antibody, an anti- oligomer antibody, which is capable of recognizing *in vitro* a structural common epitope to oligomeric conformers formed by several distinct proteins (Kayed *et al*. 2004). They studied *in vivo* the ability of A11 antibodies to neutralize the toxicity of oligomeric forms of all amyloid species on cell cultures (Kayed *et al*. 2004). They even observed that the amount of oligomer specific antibody deposition was significantly lower than the total Aβ, comprehending that oligomers do not accumulate over time, but tend to aggregate into fibrillar structures that are undetected by this oligomer specific antibody (Kayed *et al*. 2004).

In a subsequent study, Kayed and colleagues developed the OC conformational polyclonal antibody, that was able to recognize a generic epitope associated with amyloid fibrils and soluble fibrillar oligomers formed by different proteins, and that is absent in prefibrillar oligomers, specifically recognized by the A11 conformational polyclonal antibody (Kayed *et al*. 2007). Starting from these observations, the researchers hypothesized that there were at least two distinct types of amyloid oligomers: prefibrillar oligomers and fibrillar ones (Figure 17). The fact that the OC antibody recognized small soluble oligomers in the 250 kDa range of dimers that are not recognized by A11 indicates that these oligomeric species are conformationally bound to fibrils and distinct from prefibrillar oligomers (Kayed *et al*. 2007).

In 2014, Savage and collaborators developed a novel conformational antibody specific for $A\beta_{42}$ ADDLs, called 19.3. Specifically, the researchers developed a robust assay to quantify oligomers from CSF, that resulted to be significantly elevated in AD CSF compared with aged controls (Savage *et al*. 2014).

9. Anti-Aβ monoclonal antibodies approved in clinical trials for AD treatment

Over the past 25 years, several mAbs have been engineered to bind and eliminate Aβ (Karran and De Strooper 2022) from the brain of AD patients and are currently under investigation in clinical trials (Bigi *et al*. 2024b). The Aβ plaque reduction catalyzed by mAbs specific for different Aβ conformers is retained to involve the activation of microglia, following the phagocytosis of fibrillar Aβ and degradation through the endosomal/lysosomal system, as reported in Figure 18 (Cummings *et al*. 2024).

Nowadays, a mAb called Lecanemab, has received the U.S. Food and Drug Administration (FDA) approval (Swanson *et al*. 2021). However, the path to approval is often challenging. Aducanumab, which initially received accelerated FDA approval (Sims *et al*. 2023; Fletcher 2023), was subsequently withdrawn by its manufacturer, Biogen, in 2024 due to limited clinical efficacy (Alzheimer's Disease and Dementia, 2024). Another mAb, such as Donanemab (Sims *et al*. 2023; Fletcher 2023), is currently under investigation, while Remternetug is in Phase III clinical trials (Alzforum 2023). Development of Gantererumab, another mAb, was terminated after it failed to meet primary endpoints in clinical trials (Boess *et al*. 2021; Ostrowitzki *et al*. 2012).

9.1 Lecanemab

Lecanemab (BAN2401; Leqembi.) is a humanized IgG1 antibody based on the mouse mAb158 (Englund *et al*. 2007) that specifically binds to Aβ protofibrils. Lecanemab was approved in January 2023 by FDA, and it is recommended to patients with MCI or mild AD dementia with positive amyloid PET or CSF biomarkers, consistent with AD. (FDA Office of the Commissioner 2023). In phase III, Lecanemab showed to slow down the cognitive decline of AD patients compared with controls treated with placebo. (van Dyck *et al*. 2023). Furthermore, amyloid PET plaque levels were reduced in patients treated with Lecanemab, and all CSF and plasma biomarkers were decreased by Lecanemab compared to the placebo except for NfL, showing no drug-placebo difference (Cummings *et al*. 2024). Unfortunately, infusion-related reactions (26.4%), intracerebral hemorrhages (17.3%), and cerebral edema (12.6%) were the most common adverse effects (van Dyck *et al*. 2023). Furthermore, non-carriers of the APOE ε4 allele in the under Lecanemab treatment had the lowest incidence of hemorragies (11.9%) and cerebral edema (5.4%); whereas ε4 heterozygotes had a higher incidence of both (van Dyck *et al*. 2023; Cummings *et al*. 2024). Lecanemab received accelerated approval from the FDA based on its Aβ lowering activity in the Phase IIb study, which showed a reasonable likelihood of predicting clinical benefit. Based on the results of the study "CLARITY AD", Lecanemab received standard FDA approval (van Dyck *et al*. 2023).

9.2 Aducanumab

Aducanumab (BIIB037; Aduhelm™) is a humanIgG1 mAb (IgG1-mAb) specific for an N-terminal epitope composed by amino acids 3–7 of the $A\beta_{42}$ peptide, with a higher affinity for fibrillar aggregates compared with monomers (Arndt *et al*. 2018). This mAb was recommended for patients with MCI or mild dementia who were positive for amyloid PET or CSF studies (Sevigny *et al*. 2016). In phase III, Aducanumab decreased, in a dose-dependent manner, the CSF p-tau levels and plasma p-tau (Budd Haeberlein *et al*. 2022). In addition, pooled results from a small sample of patients showed a dose-dependent decrease in tau PET standardized uptake value ratio (SUVR) in precise brain regions, demonstrating that Aducanumab directly affects both an upstream biomarker of AD (Aβ plaque) and downstream biomarkers of AD (CSF and plasma p-tau; tau PET). Reductions in amyloid PET SUVR correlated with reductions in plasma p-tau 181 levels. Taken together, these results support the hypothesis that Aβ accumulation triggers downstream tau pathology and subsequent clinical decline, and that removal of aggregated Aβ in the brain via Aducanumab treatment results in clinical benefit (Herring *et al*. 2021; Chen *et al*. 2024). Unfortunately, intracerebral hemorrhages and Amyloid-Related Imaging Abnormalities (ARIA) were the most common adverse effects at high-dose of mAbs (Sevigny et al. 2016; Herring *et al*. 2021; Chen *et al*. 2024). However, after 48 months of treatment with Aducanumab, the Aβ plaques were reduced in a dose- and time- dependent manner, as visible by amyloid PET (Herring *et al*. 2021; Cummings *et al*. 2024; Chen *et al*. 2024). Unfortunately, Aducanumab has been discontinued by Biogen in 2024, due to limited clinical efficacy and the discontinuation of confirmatory trials needed for full FDA approval (Alzheimer's Disease and Dementia, 2024).

9.3 Donanemab

Donanemab (LY3002813; N3pG) is a humanised IgG1 mAb developed from mouse mE8-IgG2a (Demattos *et al*. 2012). It recognises the N-terminal pyroglutamate form of Aβ, binds to deposited Aβ plaques and activates microglia-mediated clearance (Demattos *et al*. 2012; Cummings *et al*. 2024). Several phase III studies were conducted to investigate the Donanemab treatment, and one phase III trial is complete. In patients with intermediate tau levels and clinical AD symptoms, Donanemab showed to reduce the cognitive decline by 35% in Clinical Dementia Rating – Sumb of Boxes and led to 40% less decline in activities of daily living (Eli Lilly and Company 2023). Cerebral edema appeared in 24% of participants who were treated with Donanemab, with 6.1% experiencing symptomatic edema (Cummings *et al*. 2024). Cerebral hemorrhages occurred in 31% of participants treated with Donanemab and 13% receiving placebo. Based on these results, standard approval of Donanemab by the FDA is anticipated (Eli Lilly and Company 2023; Sims *et al*. 2023).

9.4 Remternetug

Remternetug (LY3372993) is an N3pG-AB mAb, specific for pyroglutamate Aβ, and able to target Aβ plaques (Alzforum 2023). In this moment, Remternetug is tested in a phase III study, which has as primary outcome to measure the percentage of participants who reach Aβ clearance on PET, and as a secondary outcome to measure changes of Aβ plaque on PET from baseline, monitor the time necessary to reach the Aβ plaque reduction, measure of the pharmacokinetics trough serum concentration, and number of participants with treatment emergent anti-drug antibodies (Cummings *et al*. 2024). Moreover, participants who have received placebo, will be treated with Remternetug for additional 52 weeks of time, while participants treated with mAb are going to be treated with placebo (Alzforum 2023; Cummings *et al*. 2024).

9.5 Gantenerumab

Gantenerumab (RO4909832; R1450) is the first fully human IgG1 monoclonal antibody against Aβ in clinical development (Ostrowitzki *et al*. 2012). It recognizes both the N- terminal and the central amino acids of the Aβ peptide. It initiates cell-mediated clearance via the recruitment of microglia (Ostrowitzki *et al*. 2012; Cummings *et al*. 2024). Two phase III clinical trials, designated GRADUATE I and GRADUATE II, were conducted to assess the efficacy of Gantenerumab in mitigating cognitive decline in patients with early-stage AD. The results demonstrated that, although Gantenerumab was effective in reducing amyloid plaques, it did not achieve the primary endpoint of slowing clinical decline as measured by the Clinical Dementia Rating-Sum of Boxes (Boess *et al*. 2021; Ostrowitzki *et al*. 2022). An overview of these mAbs is represented in Table 3.

10. Camelid antibodies

Over the past decade, several monoclonal antibodies engineered to bind and eliminate Aβ aggregates have been developed, some of which have been applied to human trials. Despite some promising clinical data, the great majority of therapeutic trials of Aβ have so far failed to improve cognition in MCI patients (Selkoe 2019), primarily because of too late treatment and massive adverse reactions. However, the experience gained from these trials has provided important clues to enable the development of improved treatments.

In the early 1990s, antibodies composed only of the heavy chains and missing the light chains were identified in the serum of the camelid family and they were called "camelid antibodies" (Hamers-Casterman *et al*. 1993). The camelid heavy-chain antibodies (HcAbs) include only two constant domains in contrast with three constant domains present in conventional antibodies (Figure 19) (Hamers-Casterman *et al*. 1993).

Due to the lack of a light chain, the antigen binding site of heavy-chain

antibodies is formed by one single domain: the VHH domain (Hamers-Casterman *et al*. 1993) also defined as nanobodies or single-domain antibodies (sdAbs). Notably, like classical monoclonal antibodies, nanobodies exhibit high target specificity and affinity but their small size (one-tenth of a full-length IgG) makes them highly stable, soluble, able to access hidden epitopes, and functionally expressed within the cell as intrabodies (Muyldermans 2013). Their low immunogenic potential due to their high sequence homology to human and mouse V_H domains and their low molecular weight significantly reduce the intrinsic toxicity characterizing conventional antibodies, making them great tools for basic research and potential candidates for diagnostic and therapeutic purposes (Muyldermans 2013; Ackaert *et al*. 2021). Nowadays, sdAbs have been shown to be useful probes for the study of amyloid aggregation and for the prevention of amyloid- induced cytotoxicity and cell death (Aprile *et al*. 2020).

10.1 DesAb-O antibody

In the recent years, Aprile and coworkers introduced a scanning method based on the use of rationally designed single-domain (VH) antibodies (DesAbs) for sequence-activity relationship studies (Sormanni, Aprile and Vendruscolo 2015; Aprile *et al*. 2017; Sormanni, Aprile and Vendruscolo 2018, Aprile *et al*. 2020). Using this strategy, they identified the DesAb-$A\beta_{29\text{-}36}$ antibody, targeting a structural epitope formed by residues 29-36 of $A\beta_{42}$, present in oligomeric aggregates and buried in the core of the cross-β structure of mature fibrils (Colvin *et al*. 2016; Aprile *et al*. 2020), resulting in low binding of DesAb-$A\beta_{29\text{-}36}$ to these conformers, as well as to the monomeric protein (Aprile *et al*. 2017; Aprile *et al*. 2020). Therefore, they applied a rational design to generate a battery of DesAbs, targeting epitopes in the region of residues 29-36 of $A\beta_{42}$, and performing experimental screening to identify one, later called DesAb-O, that exhibited the best affinity and binding selectivity for the oligomers of $A\beta_{42}$, rather than its monomeric and fibrillar forms (Aprile *et al*. 2020) as represented in Figure 20.

Given the high affinity of DesAb-O for $A\beta_{42}$ oligomers, we investigated the ability of DesAb-O to selectively detect preformed $A\beta_{42}$ oligomers both *in vitro* and in cultured neuronal cells, by using dot-blot, ELISA immunoassay and super-resolution STED microscopy, and to counteract the toxicity induced by the oligomers, monitoring their interaction with neuronal membrane and the resulting mitochondrial impairment. We then applied this approach to CSF samples (CSFs) from AD patients as compared to age- matched control subjects (Bigi *et al*. 2024b). Moreover, in another study, with the aim of improve the outstanding ability of DesAb-O, we designed a dimeric structure of this sdAbs, adding a flexible $(GGGGS)_3$ linker to the C-terminus, allowing the protein a wide range of movement and the connection of two monomeric domains (Napolitano *et al*. in preparation).

11. Aim of the thesis

Current diagnostic criteria for AD include the use of CSF biomarkers such as the decreased CSF $A\beta_{42}/A\beta_{40}$ ratio due to $A\beta_{42}$ levels reduction, and the increased levels of total and phosphorylated tau (t-tau and p-tau, respectively). Despite their widely recognized diagnostic relevance, such biomarkers often produce uncertain results, both in terms of false positives and negatives, especially in the early AD, thus rendering the research and identification of novel biomarkers of fundamental importance for supporting the existing ones to gain sensitivity and specificity. In addition, the NIA-AA recommended a biomarker-grounded biological, rather than syndromal, definition of AD, involving biomarkers able to reflect pathophysiological changes occurring already in the early stages of AD.

All the classical protein-based biomarkers reveal the levels of specific proteins, including $A\beta_{42}$ and tau; however, as a proteinopathy, AD is characterized by a generic failure of the proteostasis network, which does not maintain proteins in a native, non-aggregated functional state. Building on this idea, we have extended our attention to the status of the entire proteome, performing an array of biophysical and cellular analyses on CSF samples from AD patients and healthy subjects, with the aim to identify new and putative biomarkers comparing their ability to discriminate between AD and non-AD cases with those of classical ones.

In this context of altered proteostasis, increasing evidence also suggests that small soluble Aβ oligomers, rather than mature Aβ fibrils, are the main agents responsible for neurotoxicity in AD. Recently, sdAbs have been proposed as promising tools for the early diagnosis and therapy for AD. Thus, the second aim of the study was to investigate the ability of DesAb-O to selectively detect $A\beta_{42}$ oligomers both *in vitro* and in cultured cells and to neutralize their associated neurotoxicity. Furthermore, we have investigated its ability to discriminate the CSFs of AD patients from those derived from age-matched control subjects by using immune-enzymatic assays and STED microscopy.

Finally, with the aim of improving the already incredible binding avidity and specificity of DesAb-O and obtain a more accurate sdAb able to detect oligomers at even lower concentrations, we have engineered the structure of DesAb-O by designing a dimeric sdAb: the Dimeric-DesAb-O. We have performed biophysical experiments to characterise its ability to interfere with the $A\beta_{42}$ aggregation process and studied its specificity and selectivity for $A\beta_{42}$ oligomers. Furthermore, we have studied the ability of Dimeric-DesAb-O to counteract the toxicity induced by $A\beta_{42}$ oligomers and CSFs of AD patients on neuronal cells, demonstrating the neuroprotective ability of this dimeric sdAb at lower concentrations as compared to DesAb-O.

Overall, this thesis aims to identify novel CSF biomarkers for AD and to explore the application of sdAbs as promising tools for the early diagnosis and therapy of AD. Overall, this research seeks to contribute to the development of more effective diagnostic tools and therapeutic interventions for a future AD cure.

12. Introduction: figures and tables

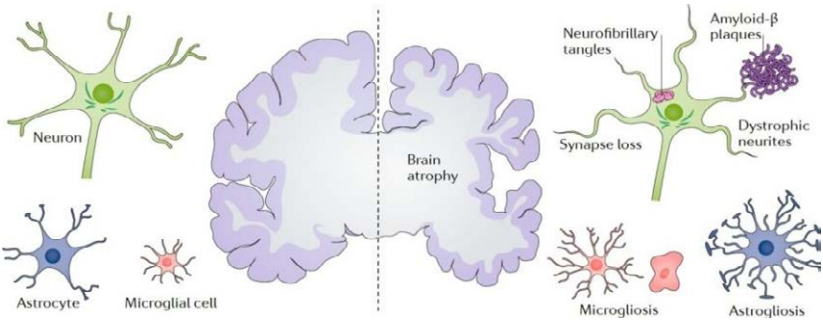

Figure 1 - Pathologic features of AD. Anatomically, AD is characterized by brain atrophy associated with loss of synapses and neurons. Microscopically, deposition of extracellular Aβ plaques and intraneuronal neurofibrillary tangles, in association with dystrophic neurites and loss of synapses, as well as microgliosis and astrogliosis (Adapted from Congdon and Sigurdsson, 2018).

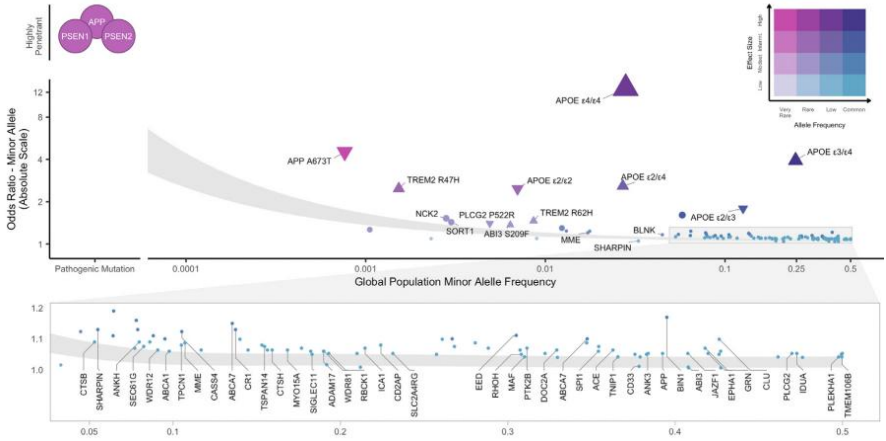

Figure 2 - Visual representation of genetic architecture of AD/dementia. This scheme illustrates the distinction between rare, high-penetrance mutations in genes such as APP, PSEN1 and PSEN2, which are strongly associated mwith early-onset familial AD, and the more prevalent variants identified by genome- wide association studies (GWAS), which confer lower risk for the more common late-onset AD. Furthermore, it shows the difficulties and possibilities inherent in elucidating the intricate genetic underpinnings of AD, as evidenced by the influence of allele frequency and effect size on the statistical capacity to identify AD-associated genetic variants (Adapted from Andrews *et al*. 2023).

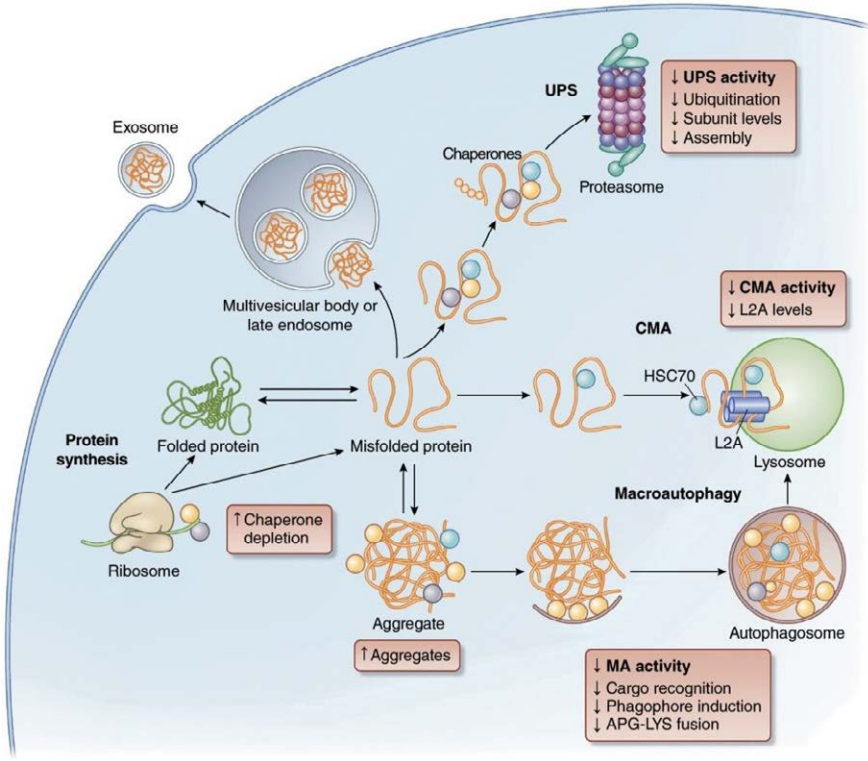

Figure 3 - Altered intracellular proteostasis systems. The intracellular proteostasis is regulated by chaperones, the ubiquitin-proteasome system (UPS) and autophagy. Chaperones, depicted as blue, yellow, and gray circles, facilitate the folding of proteins. In the event of an unsuccessful folding process, chaperones direct the unfolded protein for proteasomal (often subsequent to ubiquitination) or lysosomal degradation. In chaperone-mediated autophagy (CMA), soluble proteins may enter the lysosomal lumen via a membrane transporter. Once misfolded proteins have aggregated into oligomers or insoluble aggregates, they can only be removed from the cytosol by undergoing degradation in lysosomes via macroautophagy (MA) or via exosomes. The brown boxes indicate the alterations that occur with age in the intracellular proteostasis networks. APG-LYS: autophagosome-lysosome; HSC70: heat-shock cognate protein of 70kDa; L2A: lysosome-associated membrane protein type 2A (Adapted from Kaushik and Cuervo 2015)

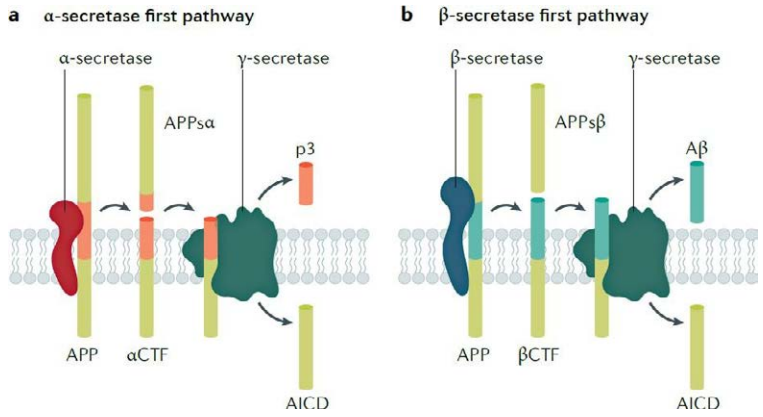

Figure 4- Proteolytic cleavage of APP. A) In the non-β-amyloidogenic pathway, APP is cleaved by α-secretase leading to the formation of sAPPα and α CTF, which in turn is cleaved by γ-secretase to produce the extracellular peptide p3 and the intracellular fragment AICD (Sewell 2007). B) Aβ is formed in the amyloidogenic pathway through cleavage of APP by β-secretase into sAPPβ and β CTF, the latter of which will be cleaved again by γ-secretase, producing Aβ and AICD (Adapted from Knopman *et al*. 2021).

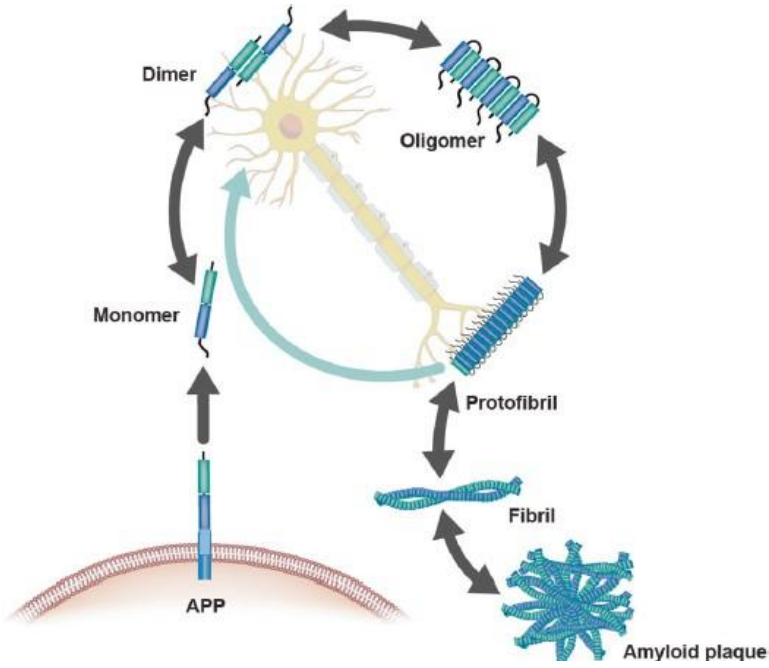

Figure 5 - Species of Aβ aggregation and evidence for reversible states: the Aβ cycle. Aβ aggregation species can exist as monomers, dimers, oligomers, protofibrils, fibrils, and amyloid plaques. These species exist in an equilibrium state in which one form can convert to another bidirectionally (Adapted from Hampel *et al*. 2021b).

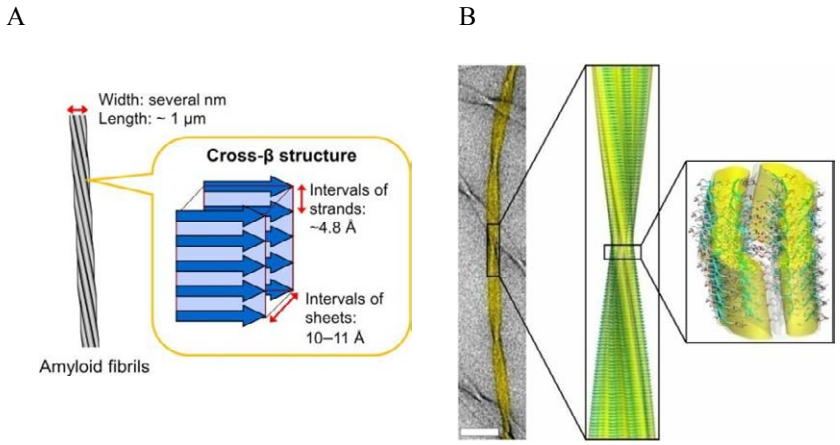

Figure 6 – Aβ fibrils structure representation. A) Schematic representation of the structure of an Aβ fibril. Aβ fibril typically have a needle-like and unbranched morphology, consisting of several protofilaments a few nanometers wide and about a micrometer long, which are bundled together laterally. Each protofilament has a cross-β structure. This means that the β strands are stacked perpendicular to the long axis of the fibril (Adapted from Chatani *et al*. 2021). B) Detailed view of the atomic resolution structure of the triplet fibril (middle). The background image of the fibril (left) was obtained by Transmission Electron Microscopy (TEM). (Scale bar, 50 nm.) The surface of the fibril (Right) is shown at 1.0 σ (white) and 2.2 σ (yellow) above the mean density, respectively, and the constituent β-sheets are shown as ribbons; oxygen, carbon and nitrogen atoms are shown in red, gray and blue, respectively (Adapted from Fitzpatrick *et al*. 2013).

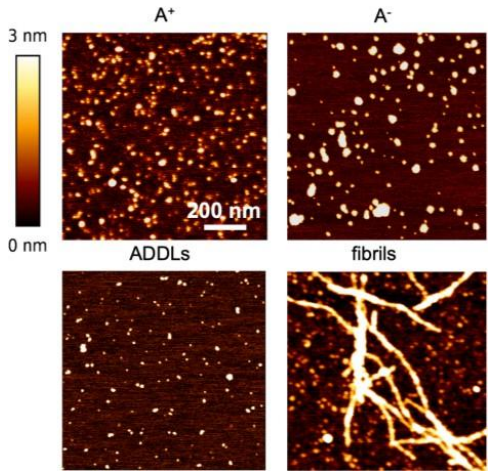

Figure 7 - AFM images of $A\beta_{42}$ oligomers (A+, A-, ADDLs) and $A\beta_{42}$ fibrils. The AFM revealing height values of 4.4 ± 2.4 nm for A+, 4.4 ± 1.5 nm for A-, 3.9 ± 1.2 nm for ADDLs (Adapted from Banchelli *et al*. 2020).

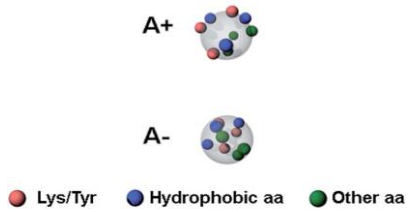

Figure 8 - Schematic picture of the A+ and A- oligomers generated from $A\beta_{42}$. Toxic A+ oligomers are characterized by exposure of hydrophobic clusters (blue), as well as of Tyr and Lys residues (red) (Adapted from Banchelli *et al*. 2020).

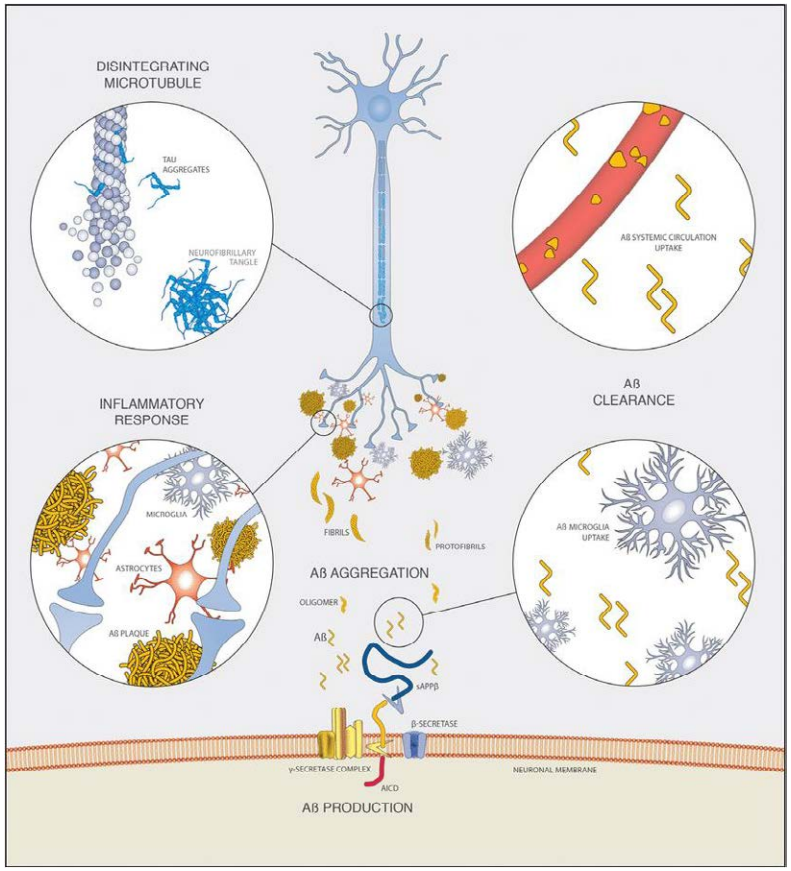

Figure 9 – Amyloid hypothesis. Visual representation of the amyloid cascade hypothesis, illustrating the key events in the development of AD: from the production and aggregation of Aβ peptides to the formation of plaques and tau-hyperphosphorylated neurofibrillary tangles (Adapted from Panza *et al*. 2019).

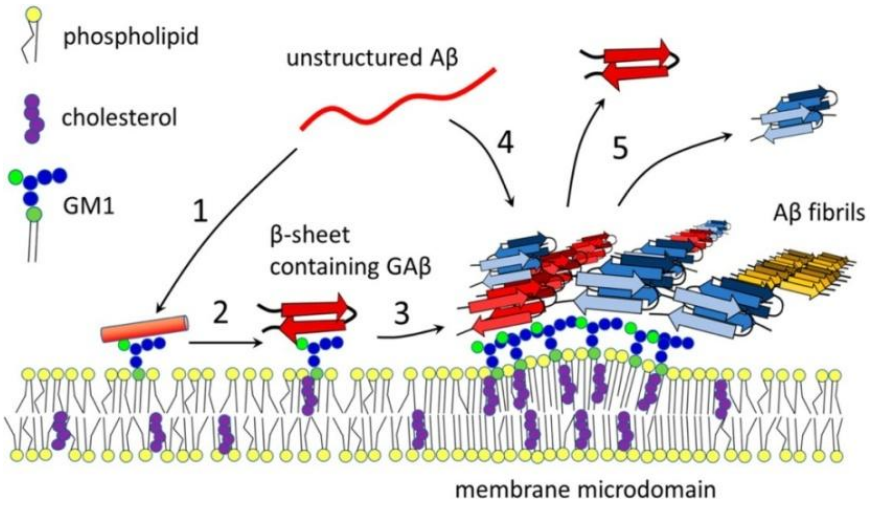

Figure 10 - Interaction Aβ/GM1. (1) α-helical conformation in Aβ is induced by not clustered. (2) Transition from α-helical to β-sheet structure induced by GM1. (3) Localized GM1 clusters in membrane microdomains are involved in increase of concentration and aggregation of Aβ peptide into higher- molecular weight aggregates. Parallel and antiparallel β structures have been found in membrane-associated amyloid fibrils. (4) Aggregation of Aβ serves as a platform for capture and binding of monomers or oligomers circulating in the intercellular space (5) (Adapted from Rudajev and Novotny 2020)

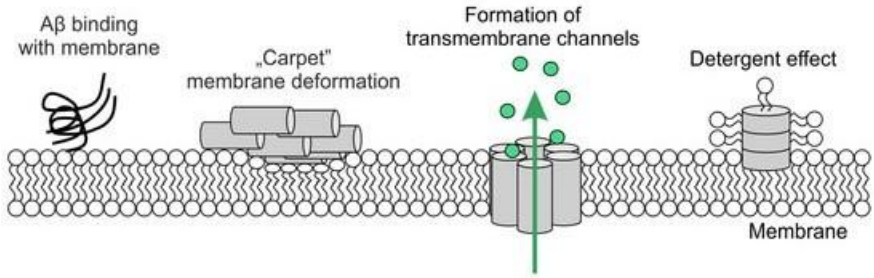

Figure 11 – Schematic representation of the Aβ-induced neurotoxicity through the formation of a transmembrane channel, via "carpet model" and the "detergent-like mechanism". (Adapted from Wiatrak *et al*. 2021).

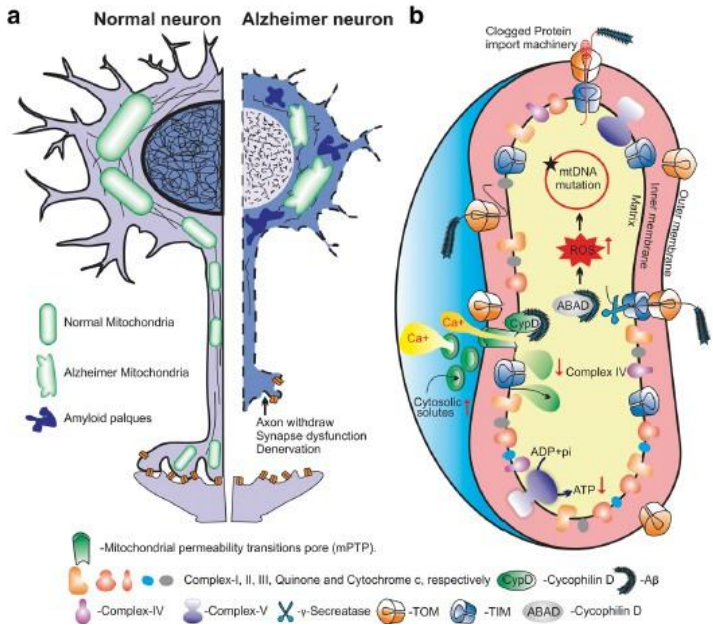

Figure 12 - Mitochondrial dysfunction in AD. A) Altered morphology of mitochondria and synaptic space and reduced level of mitochondrial axonal transport in AD neuron with respect to normal neuron. B) A detailed examination of mitochondria in a disease state reveals the translocation of Aβ into the mitochondrial matrix via the TOM/TIM complex. Following its import, Aβ disrupts mitochondrial function by: 1) impairing the activity of complex IV, 2) altering Ca^{2+} efflux, and 3) inducing mutations in mitochondrial DNA (mtDNA) (Adapted from Cadonic, Sabbir and Albensi 2016).

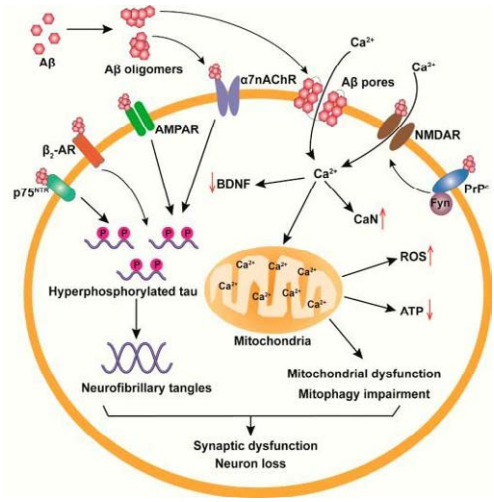

Figure 13 - Schematic diagram of neurotoxic mechanisms induced by Aβ oligomers. Aβ oligomers are found primarily at the extracellular level and can bind to multiple receptors

on the surface of the neuronal membrane, interfering with normal receptor signalling pathways. They are also able to interact directly with the membrane to form pores, leading to changes in membrane integrity and permeability. These two effects of Aβ oligomers on the cell membrane can further induce dysregulation of Ca^{2+} homeostasis, mitochondrial damage, generation of ROS, reduced ATP levels, and abnormal phosphorylation of tau, resulting in synaptic dysfunction, neuronal loss, and impaired LTP (Adapted from Huang and Liu 2020).

		Cognitive stage		
		Cognitively unimpaired	**Mild cognitive Impairment (MCI)**	**Dementia**
Biomarker profile	A-T-(N)-	Normal AD biomarkers. Cognitively unimpaired	Normal AD biomarkers with MCI	Normal AD biomarkers with dementia
	A+T-(N)-	Preclinical Alzheimer's pathologic change	Alzheimer's pathologic change with MCI	Alzheimer's pathologic change with dementia
	A+T+(N)- A+T+(N)+	Preclinical Alzheimer's disease	Preclinical Alzheimer's disease with MCI (prodromal AD)	Alzheimer's disease with dementia
	A+T-(N)+	Alzheimer's and concomitant suspected non-Alzheimer's pathologic change, cognitively unimpaired	Alzheimer's and concomitant suspected non-Alzheimer's pathologic change with MCI	Alzheimer's and concomitant suspected non-Alzheimer's pathologic change with dementia
	A-T+(N)- A-T-(N)+ A-T+(N)+	Non-Alzheimer's pathologic change, cognitively unimpaired	Non-Alzheimer's pathologic change with MCI	Non-Alzheimer's pathologic change with dementia

Table 1 – Overview of AD biomarkers continuum. Abbreviations; A, Amyloid-β; N, neurodegeneration; T, tau. Adapted from C.R.Jr., Jack, *et al*. NIA-AA research framework: toward a biological definition of Alzheimer's disease. Alzheimers Dement, 14 (4) (2018) 535-562. (Adapted from Simrén *et al.* 2023).

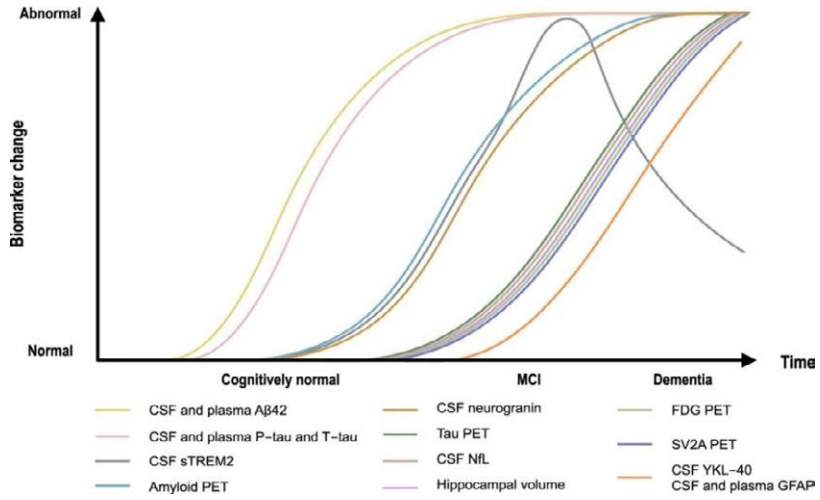

Figure 14 - A model of the temporal pattern of biomarker abnormalities for AD-related pathophysiological processes. The earliest detectable changes occur in the CSF and plasma, characterized by an altered $A\beta_{42}/A\beta_{40}$ ratio. Subsequently, amyloid plaques accumulate, becoming detectable through amyloid PET imaging. Microglial respond and secrete a soluble trigger receptor expressed on myeloid cells 2 (sTREM2), serving as a marker. sTREM2 demonstrates a characteristic peak during MCI followed by a decline upon progression to AD dementia. When tau PET becomes positive, a number of neurodegeneration and synaptic dysfunction biomarkers of function change more or less in parallel (Adapted from Zetterberg and Bendlin 2021).

SUMMARY OF ALZHEIMER DISEASE BLOOD BIOMARKERS			
	Biologic significance	**Clinical relevance**	**Limitations**
Aβ42/Aβ40[a,b]	Produced by amyloid precursor protein metabolism in brain Cleared by glymphatic system and other mechanisms Aβ42 sequestered within amyloid plaques	Reduced Aβ42 reflects brain amyloidosis Aβ42/40 ratio corrects for interindividual variation in amyloid metabolism Reduced Aβ42/40 ratio highly concordant with amyloid PET Blood and CSF Aβ42/40 highly concordant	Smaller reduction of Aβ42 in blood than CSF
Total-tau[b]	Secreted from neurons, in response to Aβ exposure or released by damaged neurons	Elevation in AD; reflects tauopathy and tangle formation Indirect marker of amyloidosis	Correlates poorly with CSF tau Does not reliably reflect tau pathology in nonAD tauopathies Elevated in stroke and CJD
Phosphorylated tau[a,b]	85 potential phosphorylation sites; several promising candidates More phosphorylation in AD	Presymptomatic elevation in AD reflects AD tauopathy and tangles Indirect marker of amyloidosis Correlates with amyloid and tau PET Blood and CSF p-tau highly concordant	Not widely available or internationally standardized
Neurofilament light[b]	Reflects rate of neurodegeneration	Reflects rate of neurodegeneration/disease progression Correlated with amyloid PET, tau PET, and MRI brain atrophy rates	Nonspecific

Abbreviations: AD, Alzheimer disease; CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; PET, positron emission tomography.
[a]Measured by immunoprecipitation mass spectrometry. [b]Measured by ultrasensitive enzyme-linked immunosorbent assay (ELISA).

Table 2- An overview of plasma biomarkers in AD. (Adapted from Zetterberg *et al*. 2020).

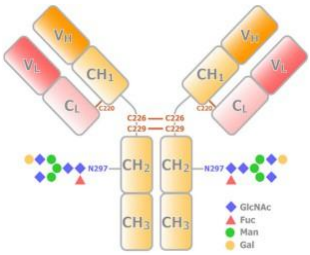

Figure 15 - Schematic representation of the structure of an IgG. IgG consist of two heavy and two light chains. Each heavy chain has VH and CH1 domains, forming the Fab portion with a light chain, and CH2-CH3 domains which are the main component of the crystallizable fragment (Fc) portion. A flexible hinge region with disulfide bridges connects Fab and Fc. The CH2 domain has an N-glycosylation site crucial for effector functions (Adapted from Tokunaga and Takeuchi 2020).

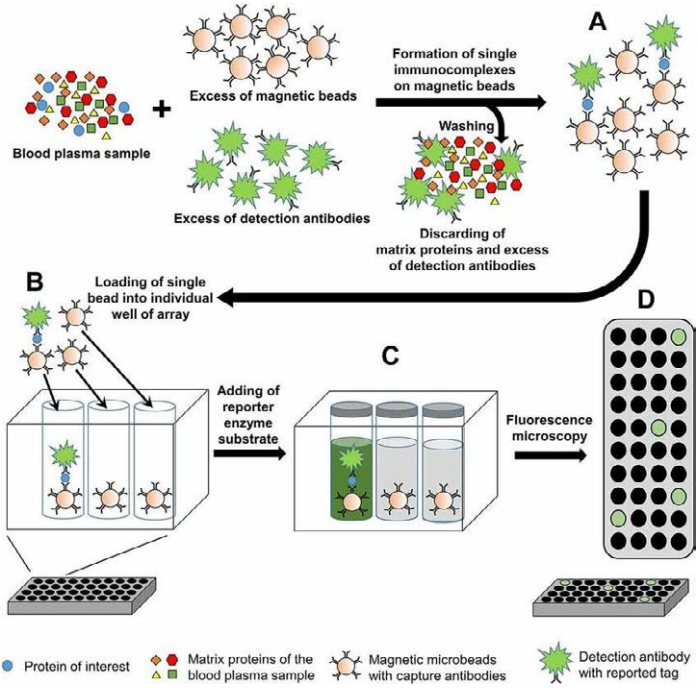

Figure 16 - The principle of SIMOA. A) A biological sample is introduced to an excess of antibody- conjugated magnetic microbeads (2.7 µm diameter), where capture and detection antibodies are immobilized on the bead surface. B) The bead-sample mixture is loaded into an array containing 50 femtoliter wells, each with dimensions of 4.5 µm diameter and 3.25 µm depth. C) Following bead loading, a reporter enzyme substrate is added, and the wells are sealed with a silicon gasket to prevent leakage and cross-contamination. D) The array is then analyzed for fluorescence signal, which corresponds to the presence and quantity of the target analyte (Adapted from Kulichikhin *et al*. 2021).

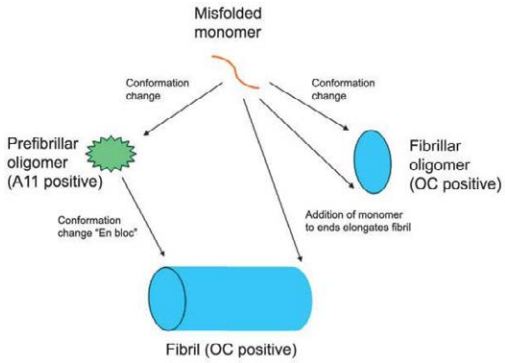

Figure 17 - Different types of amyloid oligomers and their relationships to amyloid fibrils. Amyloid aggregation involves two distinct pathways: 1) Monomers form transient prefibrillar oligomers (A11- positive, on the left) that mature into fibrils. 2) Monomers directly form fibrillar oligomers (OC-positive, on the right) serving as nuclei for fibril growth. This conformational diversity impacts our understanding of amyloid-related disease and therapeutic development (Adapted from Kayed *et al*. 2007).

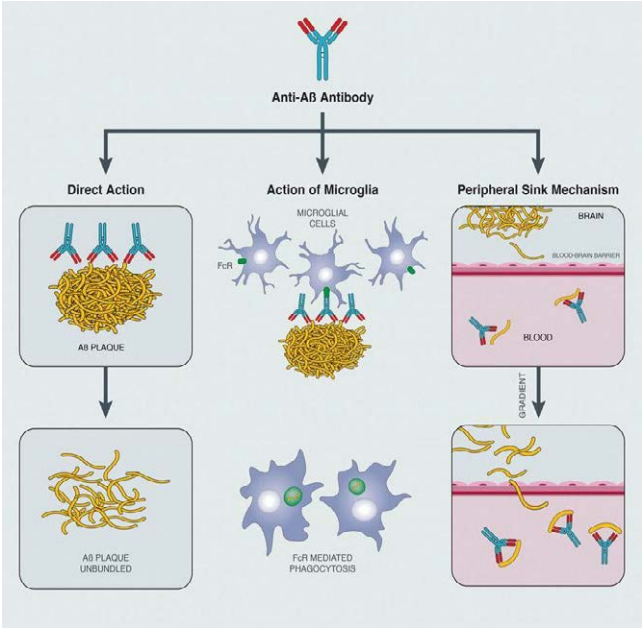

Figure 18 - Aβ removal via Aβ-specific antibodies. For the mechanism of action of anti-Aβ antibodies, there are three main hypotheses. The first is that antibody binding destabilizes different aggregate species, acting directly against Aβ plaques, fibrils, protofibrils or oligomers. The second is microglial action, leading to Fc receptor-mediated phagocytosis of Aβ. Finally, there is the hypothesis of a peripheral sink mechanism, in which the antibody

binds to Aβ present in plasma and removes it, resulting in a net efflux of Aβ from the brain to the plasma. (Adapted from Panza *et al.* 2019).

Antibody name	Antibody type	Target	Mechanism of action	Approval status	Indications	Side effects	Notes
Aducanumab (BIIB037; Aduhelm™) (Arndt et al., 2018)	Human immunoglobulin G1	Aβ aggregates	Reduction of Amyloid plaques	FDA-approved (with limitations)	Early AD (Sevigny et al., 2016)	Brain edema, brain hemorrhage (von Rosenstiel et al., 2018; Herring et al., 2021)	Discontinued by Biogen in 2024
Lecanemab (BAN2401; Leqembi) (Engund et al., 2007)	*Mouse* humanized IgG1	Aβ aggregates	Reduction of Amyloid plaques	FDA-approved (accelerated approval)	Early AD (FDA Office of the Commissioner, 2023)	Brain edema, brain hemorrhage (van Dick et al., 2023)	Ongoing clinical trials to confirm clinical efficacy
Donanemab (LY3002813; N3pG) (Tolar et al., 2020)	*Mouse* humanized IgG1	Aβ aggregates	Reduction of Amyloid plaques	FDA-approved (accelerated approval) (Eli Lilly and Company, 2023; Sims et al., 2023)	Early AD	Brain edema (ARIA), brain hemorrhage (Eli Lilly and Company, 2023; Sims et al., 2023)	Ongoing clinical trials to confirm clinical efficacy
Remternetug (LY3372993) (Alzforum, 2023)	N3pG-AB monoclonal antibody	Aβ aggregates	Reduction of Amyloid plaques	In clinical trials (Phase III)	Early AD	Under evaluation	-
Gantenerumab (RO4909832; R1450) (Ostrowitzk et al., 2012)	Fully human IgG1	Aβ aggregates	Reduction of Amyloid plaques	Failed in Phase III clinical trials	-	-	-

Table 3 – Overview of five mAbs for AD treatment.

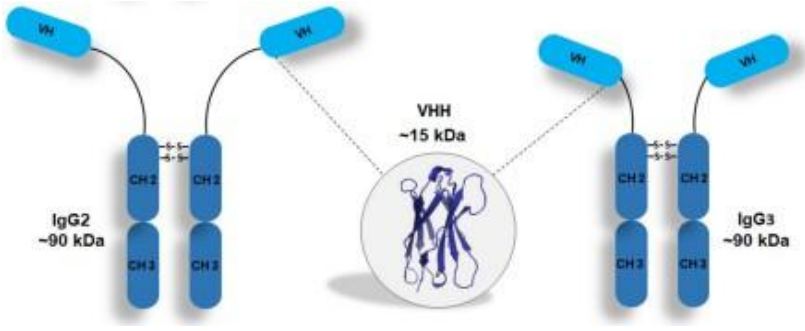

Figure 19 - Camelid antibody. The figure shows the structure of camelid antibodies: in blue are highlighted the two constant domains of the heavy chain and in light blue is highlighted the variable domain VHH (immunoglobulin G2 [IgG2] and immunoglobulin G3 [IgG3]). The three-dimensional structure of the VHH was adapted from Lesne *et al*. 2019 (Adapted from Brilhante-da-Silva *et al*. 2021).

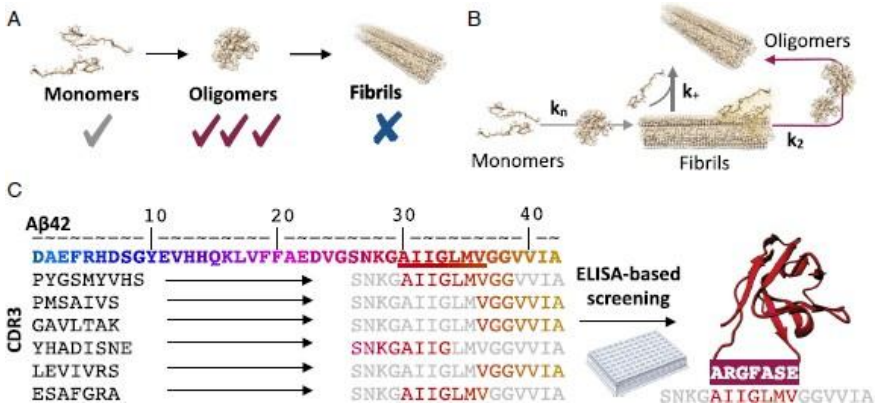

Figure 20 - Rational design of a conformation-specific antibody for $A\beta_{42}$ oligomers. A) Schematic illustration of the selections strategy to generate an antibody with selective affinity for $A\beta_{42}$ oligomers, discriminating against monomers and fibrils. B) The figure depicts the aggregation pathway of $A\beta_{42}$, including primary (k_n), secondary (k_2), and elongation (k_+) nucleation rate constants (Cohen *et al*. 2013). C) The $A\beta_{42}$ amino acid sequence is presented with a color gradient reflecting the efficacy of each residue as an oligomer binding target (red indicates high efficacy). The sequence of the complementary peptide of DesAb-O, the designed antibody exhibiting the highest affinity for Aβ oligomers, is shown in red (Adapted from Aprile *et al.* 2020).