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The AβA2V paradigm: From molecular insights to therapeutic strategies in Alzheimer's disease and primary tauopathies

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ABSTRACT

Alzheimer's disease, the leading cause of dementia globally, represents an unresolved clinical challenge due to its complex pathogenesis and the absence of effective treatments. Considering the multifactorial etiology of the disease, mainly characterized by the accumulation of amyloid β plaques and neurofibrillary tangles of tau protein, we discuss the A673V mutation in the gene coding for the amyloid precursor protein, which is associated with the familial form of Alzheimer's disease in a homozygous state. The mutation offers new insights into the molecular mechanisms of the disease, particularly regarding the contrasting roles of the A2V and A2T mutations in amyloid β peptide aggregation and toxicity. This review aims to describe relevant studies on A2V-mutated variants of the amyloid β peptide, revealing a protective effect against amyloid-β and tau pathology. Notably, special attention is given to the development of the peptide Aβ1–6$_{A2V}$(D), which shows significant neuroprotective activity through inhibition of the assembly of amyloid β into amyloid fibrils. The therapeutic potential of this peptide emerges from its ability to reduce amyloid β-induced toxicity, with promising results from studies in human neuroblastoma cells and transgenic animal models.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia worldwide. Of more than 55 million individuals who were affected by dementia in 2023, most suffered from AD. Around 10 million new cases are identified every year, and this number is anticipated to grow to 13.8 million by 2060 [1]. The prevalence of AD increases with age, with an estimated 25 %-45 % of those over the age of 85 years having dementia.

The disease is characterized by a multifaceted etiology and a progressive decline in cognitive functions, notably memory, which poses formidable obstacles to drug development. Central to its pathology is the accumulation of amyloid β (Aβ) plaques and neurofibrillary tangles of tau protein in the brain [42]. However, the precise mechanisms and their interplay remain poorly elucidated.

In both sporadic and familial cases of AD, Aβ is generated by the proteolytic cleavage of Aβ precursor protein (APP), a large type 1 transmembrane glycoprotein that consists of 695/770 amino acids [34,40,42], which primarily results in the production of Aβ with 40 (Aβ40) and 42 (Aβ42) amino acid residues [87]. In familial AD (FAD), genetic mutations in the APP or presenilin genes can result in Aβ overproduction and amino acid substitution in the central Aβ sequence, which is crucial for the protein's nucleation and folding of the generation of mutated forms of Aβ [80].

According to the "amyloid cascade hypothesis," the accumulation of aggregated Aβ has been suggested to initiate a chain of reactions that trigger and drive AD. Aβ acts as a catalyst on protein tau, triggering it to

Abbreviations: AD, Alzheimer's disease; Aβ, Amyloid β; Aβ40, Aβ with 40 amino acid residues; Aβ42, Aβ with 42 amino acid residues; Aβ1–28$_{WT}$, Aβ1–28 in the wild-type form; Aβ1–28$_{A2V}$, Aβ1–28 with A2V at position 2; Aβ1–42$_{WT}$, Aβ1–42 in the wild-type form; AβA2V, Aβ with A2V at position 2; Aβ1–42A2T, Aβ1–42 with A2T mutation; APP, Amyloid β precursor protein; AFM, Atomic force microscopy; BACE1, β-site APP-cleaving enzyme 1; Cs, Critical seed; CT, C-terminal; FAD, Familial AD; CHC, Hydrophobic core; LTP, long term potentiation; MD, Molecular dynamics; NOR, novel object recognition; NT, N-terminal; REMD, Replica exchange molecular dynamics; SPR, surface plasmon resonance; TBI, traumatic brain injury; WT, wild-type.

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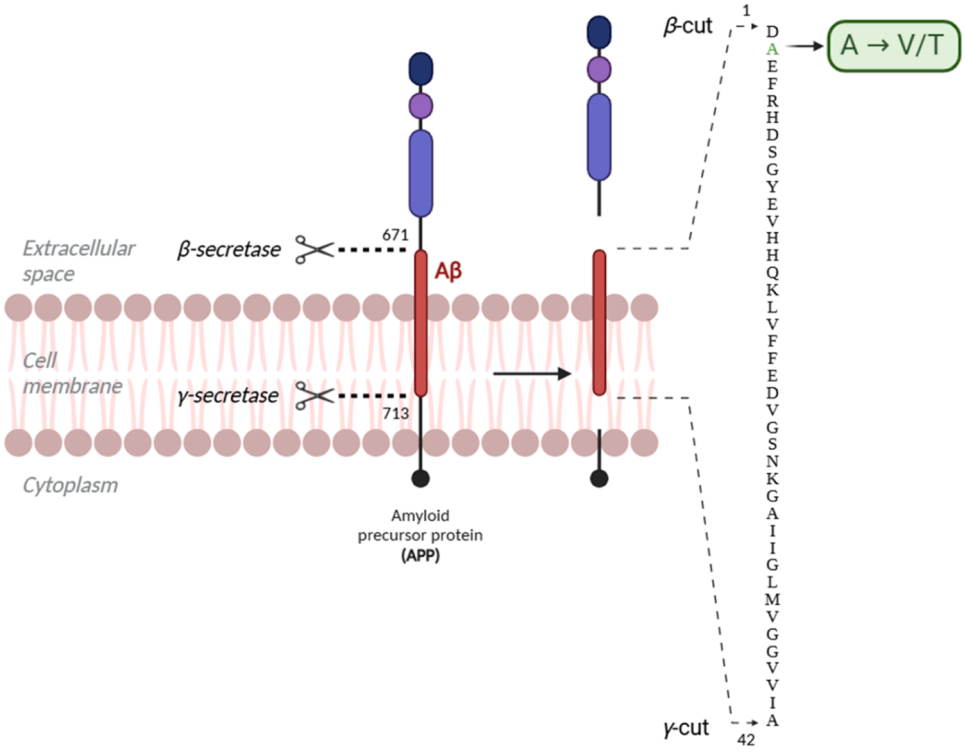

Fig. 1. Schematic representation of APP, positions of the Aβ sequence, and the alanine in position 2 of Aβ that can be substituted to valine or threonine in the presence of A673V and A673T mutations. Created with BioRender.com.

become hyperphosphorylated, unfold, and form abnormal neurofibrillary tangles. This can result in a breakdown of the neuronal cytoskeleton, significant synaptic loss, and, ultimately, neurodegeneration [89]. Recent studies, both experimental and computational, have shown that oligomeric Aβ species are among the main mediators of neurotoxicity in Alzheimer's, clarifying their interactions with cell membranes and the mechanisms that favor their pathological aggregation [73,74]. Some studies have recently suggested that Aβ and tau can act in parallel under the control of APP, which acts as a common downstream mediator [43]. This complicates the identification of effective drug targets.

Challenges mark the landscape of interventions in AD despite numerous opportunities to impede its progression [30]. Previous endeavors in clinical trials have encountered significant hurdles, with tested drugs proving either ineffective or causing severe adverse reactions [7,82]. Consequently, there persists an urgent demand for safe and efficacious drugs to combat AD. Moreover, the heterogeneity of AD exacerbates these challenges, with patients exhibiting diverse clinical phenotypes, progression rates, and responses to treatment. This variability complicates the design and interpretation of clinical trials by hampering the identification of homogeneous patient cohorts. Additionally, the absence of reliable biomarkers for early detection and disease progression further impedes diagnostic efforts and diminishes the efficacy of potential treatments.

The high failure rate in AD drug development can be attributed to several factors, including the selection of drug targets, trial design, and the translational gap between preclinical models and human disease. Most notably, drugs targeting singular aspects of AD, such as Aβ deposition, have faltered in clinical trials, emphasizing the need for more comprehensive approaches. Furthermore, clinical trials face challenges in patient selection, defining endpoints, and addressing ethical concerns, particularly given the cognitive decline in AD patients. The recent U.S. Food and Drug Administration approval of aducanumab in June 2021 [35], the first drug targeting Aβ plaques, has sparked controversy over its efficacy [81].

It is also noteworthy that a small yet significant percentage of AD cases manifest as early-onset, occurring before the age of 60–65 years. Approximately 1–6 % of all AD cases fall into this category, with a substantial portion, about 60 %, being familial. Of these familial cases, approximately 13 % appear to follow an autosomal dominant inheritance pattern, according to well-established and known research [13]. Early-onset AD is closely linked to genetic mutations in three specific genes: the APP, presenilin 1, and presenilin 2. These mutations are responsible for the abnormal production and accumulation of the Aβ peptide, leading to the formation of amyloid plaques in the brain [8,53]. In contrast, late-onset AD, which develops after age 65, has a more complex genetic background. The apolipoprotein E gene, especially its ε4 allele, is known to increase the risk of AD. However, having the ε4 allele does not guarantee the disease development, indicating the involvement of additional genetic, environmental, and lifestyle factors. Genome-wide association studies have identified several other genes associated with late-onset AD that play roles in immune response, lipid metabolism, and amyloid beta processing [91]. This underscores the importance of understanding the genetic underpinnings of AD, particularly in the context of early-onset cases, for better targeted interventions and improved patient care.

2. DISCOVERY OF THE A673V MUTATION ON APP GENE

In 2009, researchers from the Istituto Neurologico Carlo Besta of Milano discovered a new genetic mutation in the APP gene that only causes disease when present in the homozygous state [25]. The mutation involves a substitution of alanine with valine at position 673 (following the numbering of APP770) (A673V), which corresponds to position 2 of Aβ, named $A\beta_{A2V}$ [25] (Fig. 1). This genetic defect was detected in a 36-year-old patient with early-onset dementia and in their younger sister, who experienced mild cognitive impairment in multiple domains [25]. Six other family members who carry the A673V mutation in the heterozygous state were unaffected, as confirmed by formal neuropsychological assessment. This is consistent with the inheritance pattern of a recessive Mendelian trait.

Heterozygous carriers expressed both mutated and wild-type APP mRNA [25].

The patient with the A673V mutation exhibited behavioral changes and cognitive deficits. The condition progressed to severe dementia with spastic tetraparesis over approximately 8 years, and the patient died at age 46. Cortico-subcortical atrophy was observed through serial magnetic resonance imaging. Cerebrospinal fluid analysis revealed decreased Aβ1–42 levels and increased total 181T-phosphorylated tau levels, like those usually observed in elder AD patients suffering from sporadic AD. The plasma of the patient and his homozygous sister had higher levels of Aβ1–40 and Aβ1–42 than non-demented controls, while the six A673V heterozygous carriers had intermediate levels [25]. It was noteworthy that A673V, unlike the other familial mutations, causes AD only in the homozygous condition [25].

The neuropathological aspect of AD associated with the recessive APP A673V mutation was further investigated [37]. Histopathological features of the A673V carrier's brain were compared with those of a patient with FAD who died at the same age of 46 years, and distinctive features within the cerebral cortex were observed. Notable features included diffuse neuronal loss, astrogliosis, and microglial activation. At the same time, neuropil vacuolization characterized the parenchyma of the A673V carrier, with amyloid deposits abundantly distributed in the vessel walls. Amyloid plaques, particularly in the cerebral cortex and cerebellum, were notable for their unusually large size, exceeding 150 μm in diameter [37]. Staining methods revealed an overlap of Aβ1–40 and Aβ1–42 in these deposits, showcased through various tentorial methods, indicating a unique architecture not observed in other FAD forms. All the lesions show the same architecture and correspondence between the four tentorial methods used for recognition, namely thioflavin S, 4G8 antibody against total Aβ, and specific antibodies recognizing Aβ1–40 or Aβ1–42. Immunostaining for phosphorylated tau uncovered diffuse neurofibrillary changes in the cerebral cortex. However, unlike these changes, the amyloid deposits exhibited atypical characteristics, appearing large and perivascular. The authors also shed light on the topographic distribution of Aβ deposits, noting a distinctive sparing of the neostriatum while affecting the thalamus, cerebellum, and brain stem. This unique distribution deviates from established hierarchical patterns identified in sporadic AD [37].

In a broader context, the research contributed to understanding phenotypic variability linked to APP mutations. The A673V mutation presents unprecedented characteristics compared to other reported cases of cerebral Aβ amyloidosis associated with APP mutations. Thus, the study offered a nuanced and detailed exploration of the neuropathological intricacies of the A673V APP mutation and its implications for AD.

In 2012, a team of researchers led by geneticist Kari Stefansson discovered another mutation at codon 673 of the APP gene, which resulted in the amino acid substitution of alanine with threonine (A673T) (Fig. 1) [48]. Although A673V and A673T substitutions are adjacent to APP's aspartic acid protease cleaving enzyme, the resulting mutations seem to exert opposite effects on Aβ assembly. Insertion of the A673T mutation produces a robust protective effect against AD and cognitive decline in older people without AD in an Icelander cohort. The genomes of over 1800 Icelanders were analyzed, including those with and without AD, and it was found that only 0.5 % of those with AD had the A673T mutation, compared to 5 % of those without the disease. The study also found that carriers of the A673T mutation had a 40 % lower risk of developing AD, better cognitive function, and lower brain shrinkage levels than noncarriers [48]. These findings strongly supported the active role of the Aβ N-terminus region in driving AD pathology.

3. CHEMICO-PHYSICAL STUDIES ON A2V-MUTATED Aβ PEPTIDES

3.1. *In Vitro Studies*

To understand whether A673V and A673T mutation in APP, resulting in A2V and A2T substitution in Aβ, can directly affect protein production, assembly, and toxicity, numerous biochemical and biophysical studies have been performed by independent groups. In particular, through a combination of *in silico* studies, X-ray and neutron diffraction experiments, polarized light microscopy, and atomic force microscopy (AFM), scientists have offered a logical explanation for the paradoxical effects of the contrasting A2V and A2T mutation.

The effects of punctual A-to-V mutation at position 673 of the APP exerted opposite effects on the onset of AD, on the Aβ aggregative properties, interaction with membranes, and association with the onset of AD [20]. In particular, the A2V mutation enhanced APP processing, increasing Aβ production, compared to the APP wild-type.

Studies performed to understand the influence of the A673V mutation on Aβ folding show that Aβ1–42 carrying the A2V mutation ($A\beta1–42_{A2V}$) followed a pathway of structured oligomer formation different from that of Aβ1–42 in the wild-type form ($A\beta1–42_{WT}$), with early assemblies leading to efficient aggregation [67]. Similar results were obtained in a study focused on the kinetic behavior of the A2V-mutated Aβ variant and its implications in AD [66]. The aggregation kinetics of $A\beta1–42_{A2V}$ was compared to that of $A\beta1–42_{WT}$ and indicated that the mutation causes a shift in the reactive flux toward a secondary pathway, leading to a higher dominance of oligomer generation through nucleation of monomers on the fibril surface. In addition, the combined rate constant of elongation and secondary nucleation was significantly higher for $A\beta1–42_{A2V}$ than for $A\beta1–42_{WT}$. Moreover, the critical seed (Cs) concentration required for the aggregation of $A\beta1–42_{A2V}$ is lower than that of $A\beta1–42_{WT}$, indicating a more substantial seeding effect and a higher efficiency of secondary nucleation. The increased dominance of secondary nucleation over primary nucleation was attributed to an increased hydrophobicity caused by the A2V substitution, suggesting that the hydrophobic effect strongly drives this process [66]. The higher hydrophobicity of mutated oligomers compared with $A\beta1–42_{WT}$ was confirmed by spectral analysis, suggesting that this characteristic can result in an increased toxic propensity. Small-angle X-ray scattering experiments also revealed a different spatial arrangement in the structured oligomers formed by wild-type and mutated peptides [67]. Additionally, a modulating effect of pH on electrostatic interactions was noted, with a shift from pH 8.0 to pH 7.4 resulting in increased affinity due to attenuation of electrostatic repulsion among neo-formed monomers and between monomers and fibrils [66]. In addition, studies performed on Aβ1–40 revealed that the A2V substitution accelerates peptide fibrillization [57].

The structural and morphological features of the different Aβ peptides at various stages of aggregation were also characterized by using techniques such as static and dynamic laser light scattering and circular dichroism [20]. In particular, in the early stages, the aggregation process of $A\beta1–42_{WT}$ and $A\beta1–42_{A2V}$ focused on soluble oligomers and structured oligomers, which are crucial for their toxic potential. Distinctive pathways of monomer aggregation were observed between the two peptides, including differences in kinetics, the extent of the process, the size of aggregates, and the evolution of secondary structure. This suggested that the peptides exhibit different propensities to interact with similar molecules, potentially leading to oligomeric species with varying reactivity. The time evolution of aggregate collections indicated that intermediate aggregates did not uniformly progress toward prefibrillar and fibrillar structures, with fibrils forming randomly in solution and precipitating rapidly [20]. Additionally, $A\beta1–42_{A2V}$, in their early stages of aggregation, exhibited a higher propensity to interact with model membranes than $A\beta1–42_{WT}$, leading to a decrease in lipid core density and an increase in the disorder of chain packing within the membranes

[20]. Altogether, these findings indicate that A2V mutation enhances the propensity of Aβ to form oligomers and aggregates and that this can underly its higher toxicity compared to the $Aβ1–42_{WT}$.

The role of the A2V mutation in protecting heterozygous carriers was then investigated [67]. Co-incubation of $Aβ1–42_{WT}$ and $Aβ1–42_{A2V}$ led to both globulomers, annular structures, and smaller oligomers, suggesting a lower propensity for oligomerization than the $Aβ1–42_{WT}$ alone. In addition, the mix of wild-type and mutated Aβ1–42 peptide had a delayed and slower aggregation process than $Aβ1–42_{WT}$ [67].

The N-terminal (NT) Aβ residues play a role in interfibrillar interactions [85] and metal coordination [88]. In A673V homozygous carriers, the valine-valine interactions in mutated Aβ may encourage fibril polymerization and interaction with adjoining fibrils, as compared to alanine-alanine interaction in nonmutated individuals [14]. Thus, the A2V mutation promotes the adoption of a β-sheet structure by Aβ. increases oligomer formation and amyloid fibril production.

The different responses to treatments observed in clinical trials were suggested to be linked to the polymorphism of Aβ fibrils and their correlation with various neuropathological and biochemical profiles. To recognize the diverse subtypes of AD, Cantu' and colleagues [14] examined the diffraction patterns of Aβ1–28 in the wild-type ($Aβ1–28_{WT}$), A2V-mutated form ($Aβ1–28_{A2V}$) and their mixture (1:1 molar ratio). These peptides were dried slowly under a static magnetic field, morphologically characterized under different conditions, including as-deposited samples, and reconstituted in an aqueous solution. Diffraction patterns from lyophilized $Aβ1–28_{WT}$ and $Aβ1–28_{A2V}$ peptides revealed characteristic features of β-sheet conformation, similar to fibril-forming fragments of Aβ. Analysis indicated that both peptides assembled into fibrils with similar local packing and sizes. To enhance the macroscopic axial orientation of amyloid fibrils, concentrated solutions of the peptides were aligned while drying under a magnetic field. $Aβ1–28_{A2V}$ fibrils exhibited high alignment, while $Aβ1–28_{WT}$ and the mixtures showed two populations of fibrils oriented at an angle ~30°-40° to one another. X-ray and neutron scattering analysis confirmed differences in the mesoscale organization of the fibril assemblies, with $Aβ1–28_{A2V}$ showing sharp, highly oriented scattering compared to $Aβ1–28_{WT}$ [14]. The scattering patterns also revealed differences in the samples' shape and angular distribution of intensities. $Aβ1–28_{A2V}$ exhibited well-formed, highly oriented fibers, while $Aβ1–28_{WT}$ showed less well-oriented fibers with stronger lateral interactions. A mix of $Aβ1–28_{WT}$ and $Aβ1–28_{A2V}$ displayed varying degrees of organization, with broad and crisscross scatter [14]. Further analysis indicated differences in the fibril assemblies' widths and the spatial arrangements of structural units. $Aβ1–28_{A2V}$ fibrils comprised smaller, more tightly packed units than $Aβ1–28_{WT}$, suggesting a more condensed structure. These structural differences were confirmed by AFM experiments with $Aβ1–28_{A2V}$ forming large macro-fibrillar complexes and $Aβ1–28_{WT}$ displaying twisted macrofibrils. Mixtures showed a combination of twisted and single fibrils, with variations in size and morphology depending on peptide composition [14]. Hydrogen-deuterium exchange experiments revealed differences also in the propensity of fibrils to interact with solvent, with $Aβ1–28_{A2V}$ fibrils exhibiting slower exchange rates and greater protection of hydrogens within the fibril structure [14]. In conclusion, these results demonstrated that the NT region is critical in Aβ assembly. The A2V mutation promotes a unique oligomerization pathway, leading to annular structures with potentially higher toxicity than wild-type proteins. The mutation hinders aggregation in the heterozygous state by disrupting stable intermolecular interactions with the wild-type sequence, thus exerting a protective effect. These studies also provided insights into the structural diversity of fibrils formed by A2V-mutated and wild-type Aβ and their potential implications for AD pathogenesis, highlighting the importance of understanding subtype-specific characteristics in developing targeted treatments for AD.

Due to the relevance of the NT region in Aβ misfolding, the A2T mutation has also been the focus of growing interest in neuroscience research. The A2T substitution in APP, differently from A2V, resulted in similar levels of the Aβ output compared to the APP wild-type [20]. However, A2T mutation significantly impacts the stability of monomeric Aβ and its aggregation properties, potentially altering the course of AD pathogenesis [12,20,57,63,68,76,96]. Some studies highlighted that the A2T mutation may affect the biological properties and morphological characteristics of Aβ aggregates differently from the A2V substitution. Still, agreement on the impact of A2T substitution has yet to be achieved. Experiments performed in cell-free conditions have reported a slight increase in aggregation propensity when A2T substitution is inserted in Aβ1–42 but not in the Aβ1–40 peptide [12]. By contrast, other studies have shown that Aβ1–40 and Aβ1–42 carrying the A2T mutation aggregate slower than wild-type and A2V-mutated peptides [63,68,76,96].

Monomers of Aβ1–42 carrying the A2T mutation ($Aβ1–42_{A2T}$) exert lower stability than those with the A2V substitution [68]. This difference in stability is observed in various biophysical behaviors, including aggregation kinetics, primary nucleation, the abundance of oligomers, and extended conformations. In addition, aggregation reaction modeling predicts the reduced stability of the $Aβ1–42_{A2T}$ monomer, corroborated by ion mobility spectrometry-mass spectrometry measurements. Unique morphologies of $Aβ1–42_{A2T}$ aggregates were observed using AFM, potentially explaining the reduced inhibition of the long-term potentiation (LTP) of hippocampal cells compared to $Aβ1–42_{A2V}$ and $Aβ1–42_{WT}$. Murray et al.'s [68] findings suggest that the difference in stability observed between the $Aβ1–42_{A2T}$ and $Aβ1–42_{A2V}$ monomers and the distinct aggregate morphology of $Aβ1–42_{A2T}$ could contribute to explaining the different effects observed in patients carrying the two mutations.

Differently from A2V, the A2T substitution in Aβ1–40 retards peptide fibrillization [57]. Additionally, NT fragments of $Aβ1–40_{A2T}$ ranging from residue 1 to residue 7–10 retard the fibrillization of $Aβ1–40_{WT}$, highlighting a novel targeting site for the potential therapeutic development of AD [57].

In the early stages of aggregation, A2T mutation could significantly alter the β-hairpin population and shift the equilibrium toward alternative structures. The time evolution of aggregate collections indicated that intermediate aggregates did not uniformly progress toward prefibrillar and fibrillar structures, with fibrils forming randomly in solution and precipitating rapidly [20]. Similarly to $Aβ1–42_{A2V}$, the $Aβ1–42_{A2T}$ peptide exhibited a propensity to interact with model membranes, decreasing lipid core density and increasing the disorder of chain packing within the membranes [20]. It is important to point out that although recent computational studies have shown that the A2T mutation does not significantly alter the stability of the β-barrel tetramer of Aβ1–42 in a lipid membrane context, it does contribute to changes in the toxic properties and aggregation dynamics of the peptide compared to the wild-type sequence and other mutations, suggesting a unique mechanism of neuroprotection [72]

The observations gathered by these studies suggest that the heterozygous A2V and A2T-mutated Aβ can stabilize specific protein conformations, thus reducing aggregation and the formation of cytotoxicity species. In addition, they highlight the complex interplay among specific mutations in the APP, the aggregation dynamics of Aβ peptides, their toxicity, and their interaction with membranes, shedding light on the complex mechanisms underlying the pathogenesis of AD.

3.2. *In silico studies*

Molecular dynamics (MD) simulations have been employed to characterize the conformational and energetic landscapes of Aβ peptides and to develop an understanding of the experimental observations of the effects of mutations, including A2V, on the pathogenicity and aggregation propensity of Aβ peptides.

Here, we review the literature on MD simulations of Aβ peptides focused on rationalizing the differences between WT and the A2V

mutant. More general reviews of molecular simulation studies of broader sets of Aβ mutants can be found in the literature [45,70]. The effect of point mutations has been studied mostly using atomistic models, which are naturally able to resolve the interactions between the moieties affected by point mutations.

These models have provided a high-resolution insight into the structural changes and interactions but, due to their computational cost and spatiotemporal limitations, have been limited to monomeric and dimeric systems.

To overcome the daunting task of sufficiently exploring the conformational landscape of intrinsically disordered peptides with atomistic simulations, sampling both chain conformations and intermolecular interactions, many groups have adopted enhanced sampling approaches, among which replica exchange molecular dynamics (REMD) is the most common. REMD allows exploring rugged free energy landscapes and overcoming high free energy barriers, effectively accelerating conformational sampling.

3.2.1. Mutation impact on the monomer conformation

Studies revolving around monomeric systems have provided precious insight into the conformational behavior of Aβ peptides, allowing characterization at the atomistic level of the different interactions and the global structural effects due to single-point mutations in the limit of diluted solutions [25]. However, these studies are limited in the explanation of 'paradoxical' effects that can arise in systems at finite concentration, as is the case of the A2V mutation, which has a different impact on the phenotype of AD in heterozygous and homozygous states [25].

Studies by Di Fede et al. Di Fede et al., [23], Kennedy-Britten et al. Kennedy-Britten [50], and Nguyen et al. Nguyen et al., [77] used atomistic MD simulations to explore the local effects of the A2V mutation, focusing on the modeling of shorter versions of the Aβ peptide. Di Fede et al. Di Fede et al., [23] looked at the effect of the point mutation on the overall size of the Aβ1–6 peptide employing brute force MD simulations, showing that the mutated variant is more compact, with a smaller radius of gyration. This is rationalized in terms of a change in secondary structure and intramolecular interactions. Notably, the mutation of alanine to valine in position 2 induces an increase of β-turn conformations, which disrupts the formation of salt bridges between charged and polar residues in NT residues. These effects are consistent with those observed by Kennedy-Britten et al. Kennedy-Britten [50], who studied the differential behavior of single-point mutants of Aβ1–16, including A2V. The authors show that this mutation decreases the frequency of salt bridges that involve residues close to the point of mutation, effectively impacting the local structure of the peptide. For longer peptides, like in the case of Nguyen et al., who studied the effect of the mutation in the Aβ1–28 peptide, global effects such as overall size and secondary structure population are less evident [77]. Nonetheless, it is possible to observe important local effects, even at positions that are distant from the site of the mutation. In fact, the authors reported that this mutation weakens long-range contacts between the NT region and residues 22–28 when compared to WT.

Two studies have focused on the effects of single-point mutations on Aβ1–40 and Aβ1–42 peptides on the conformational landscape of the monomers [22,60]. Das et al. Das et al., [22] showed that even though the point mutation does not alter the intrinsically disordered nature of the Aβ1–42 peptide, it affects locally the secondary structure propensity. In particular, the A2V mutation induces an increase of β-structure content compared to wild-type within the central hydrophobic core (CHC) and C-terminal (CT) region, giving the mutant a more robust nature of β-hairpin interactions. The authors characterized the conformational landscape as a function of two variables, namely, the number of contacts between the NT and CHC regions and the number of contacts between the CHC and CT regions. Indeed, conformations with large values of both variables are stabilized in the mutated peptide compared to WT [22]. The higher frequency of long-range interactions involving the NT region and the rest of the peptide was confirmed by Liu et al. Liu et al., [60]. The authors analyzed the spatial organization of the domains of Aβ, showing that interaction of the mutated NT region may affect the sampling of some tertiary structures, with a possible repercussion on the aggregation propensity of A2V.

A series of studies by the group of Jin Yong Lee compared the different histidine tautomers in WT and A2V Aβ1–42 and Aβ1–40 peptides [55,56,84]. The authors evidenced that the effect of the mutation on the secondary structure content, in particular of β-sheets and α-helices, upon mutation can significantly vary as a function of the tautomeric state of the three histidines that are present in the NT region, with a general propensity for an increase of β-sheet content and a decrease of α-helix content upon mutation [56]. Also, the intramolecular interaction pattern varies for different histidine protonation states, indicating a possible cooperative effect of mutations and histidine tautomerism in the folding of Aβ peptides [55,56].

The alanine to valine mutation also affects the overall hydration of the peptide, as shown by the group of Parbati Biswas [2–4]. In particular, the mutation affects the hydration volume of the peptide, defined as the change in the solvent volume due to the solvent molecules' interaction with the surface-exposed charged and polar groups of the protein. The authors found a correlation between this quantity and the propensity to form aggregates, which can be rationalized in terms of increased hydrophobicity characteristics of the peptide upon mutation. In fact, this leads to lower survival probabilities of the hydration shells around the A2V mutant compared to the WT [4].

Finally, atomistic models have been used to evaluate the stability of monomers in the presence of curcumin [10], a small molecule with the ability to inhibit aggregation of Aβ peptides [31,61,78,92], and in presence of hexapeptide amyloid inhibitors [17]. Awasthi et al. Awasthi et al., [10] compared the conformational landscape of WT, A2V, and A2T mutants upon inhibitor binding, concluding that the A2V mutant is the most stable of the three in the absence of curcumin, while the opposite is true for A2T, consistently with the different aggregation propensities of the two mutants.

The study by Chakraborty and Das [17] marks a first step in the study of intermolecular interactions between Aβ peptides; they performed simulations of one $A\beta1–42_{WT}$ in the presence of mutants of the Aβ1–6 peptide, including A2V. The authors found that the presence of the peptides alters the tertiary structure of the $A\beta1–42_{WT}$ peptide. In particular, in the presence of the A2V hexapeptide, unstructured conformations of $A\beta1–42_{WT}$ are stabilized, sequestering the CHC and CT regions and thus eliminating the structural features essential to aggregation.

3.2.2. Mutation impact on intermolecular interactions and oligomer formation

The impact of mutations on the collective assembly behavior of disordered peptides, and ultimately on the emergent pathological aspects of amyloid aggregations, is crucially mediated by intermolecular interactions. Molecular simulations capable of shining light on the aggregation behavior of mutants with atomistic detail remain computationally inaccessible. However, advances in algorithm development and computational platforms have enabled investigation of the role of mutations in the assembly of the Aβ peptides by allowing simulations on the smallest possible aggregate units, i.e., dimers, with atomistic resolution in explicit aqueous environments.

While far from being directly able to provide detailed information on the aggregation pathway, the choice of dimers can lead to quantitative insight into the modulation of intermolecular interactions caused by point mutations.

In this field, the work of the Derreumaux group has contributed decisively, carrying out and analyzing studies of the stability of (proto) fibrils formed by homotypical and heterotypical peptides [83]. These studies focused on the impact of changes in the interactions between monomers and their relations to the dimer conformational landscape in

solution [75,76].

The challenge posed by complex conformational landscapes to developing reliable atomistic models of oligomeric structures is related to the inherent complexity of the free energy surface underpinning such transformations. In this context, Belfort and coworkers used REMD to assess the interactions between NT regions of Aβ peptides, while the CHC and CT regions were restrained to match their NMR structure [83]. Their study highlighted that peptides containing the A2V mutation tend to form more contacts compared with the homozygous WT peptides. Moreover, even if A2V-A2V and WT-A2V can establish a similar number of contacts between residues, two-dimensional intermolecular Cα distance maps show that the WT-A2V dimer forms a distinct interaction network with respect to WT-WT and A2V-A2V systems, which are instead similar. While this result alone does not explain the protective effect of the A2V mutations in heterozygous systems, it indicates that the asymmetry in the interaction patterns that leads to protective effects has a molecular-level signature in the intermolecular interactions.

Qualitatively similar information was gathered by Derreumaux and coworkers, who studied the effect of the A2V mutation in Aβ1–40 dimers [75,76] in unconstrained REMD simulations for WT-WT, WT-A2V, and A2V-A2V dimers. Their analysis of intermolecular side-chain contacts indicates that the homozygous A2V mutation increases the contacts between NT regions compared to the WT-WT system. At the same time, the heterozygous state (WT-A2V) induces a loss of intermolecular contacts, which are compensated with an increased tendency to form intramolecular contacts. Derreumaux and coworkers applied approximate methods such as MMPBSA, MMGBSA, and MM-3DRISM to quantify the energetics associated with a different intermolecular interaction pattern. Despite significant uncertainties with all these methods, the energy ranking of different systems is consistent, and A2V-A2V is the most stable dimer, followed by WT-WT and then by WT-A2V. Remarkably, the same study also compared the energetics of the protective homozygous mutation A2T, which ranks between WT-WT and WT-A2V. These observations suggest that, indeed, a careful analysis of dimer stability can yield a signature of the protective effect of a given point mutation.

Finally, atomistic simulations provide insight into the secondary and tertiary structures of dimers. While all systems are predominantly disordered, Derreumaux and coworkers note that highly populated β-hairpin conformations are found within the CHC and CT regions.

An analysis of tertiary structures shows that a dominant configuration emerges only in the heterozygous case, where the WT-A2V system displays a three-stranded β-sheet spanning the NT, CHC, and CT regions and involves residues that are experimentally known to participate in the Aβ aggregation and oligomers formation. Generally, point mutations affect the partitioning of intra and intermolecular interactions, and mutations leading to a more pronounced intramolecular stability hinder the dimerization process. This simple but crucial observation provides an additional molecular basis for understanding the protective effects of the A2V mutation that emerge when symmetry is broken and A2V monomers coexist with WT chains.

3.2.3. Computational studies of amyloid aggregation

While a direct investigation of the emergent collective behavior of multiple peptide chains undergoing an aggregation process is far from practical at the atomistic level, coarse graining strategies have been applied to study the nucleation of Aβ protofibrils by Wolynes and coauthors [95].

In their study, Zheng et al. used a coarse-grained approach that captures solvent effects as well as key elements of the secondary structure of peptides, in conjunction with umbrella sampling simulations to obtain a computational estimate of the nucleation free energy for several mutations of the Aβ peptide. Their study included the A2V mutant in its homotypic configuration. By correcting for finite-size effects in calculating nucleation barriers, the authors demonstrated that nucleation of the homozygous A2V mutant is associated with lower free energy barriers than its WT counterpart [95].

When related to the results obtained from single chain and dimers simulations, their work provides a complete perspective on the effects of a point mutation on the conformational ensemble of the Aβ peptide in solution, thus demonstrating that point mutations lead to differences in the partition of intra and intermolecular interactions (which in turn affect the conformational motifs as well as collective assembly processes) and providing a molecular-level rationale for *in vitro* and possibly *in vivo* experimental observations.

4. BIOLOGICAL EFFECTS OF THE A2V MUTATION

Turning to the impact of the A673V mutation, human brain tissue from homozygous carriers has illuminated its role in significantly elevating Aβ production, fostering oligomer and amyloid fibril formation, and intensifying neurotoxicity. Numerous studies have been performed *in vitro* and *in vivo* to elucidate the mutation's biological impact and the mechanisms underlying its pathological role.

4.1. In Vitro Studies

In AD patients, proteasomal activity has been demonstrated to be compromised, leading to neuronal death and Aβ accumulation [49,62]. Specifically, inhibiting the 26S proteasome reduces Aβ degradation [62]. Some evidence supports a direct interaction between the core 20S proteasome and Aβ peptides that leads to an inhibition of proteolytic activity, possibly contributing to the pathogenesis of AD [41,62].

Our group recently assessed the influence of mutations on proteasomal activity in human neuroblastoma SH-SY5Y cells stably transfected with human APP in the WT form or carrying the K670M/N671L substitutions, known as Swedish, A673T, and A673V mutations.

Additionally, the influence of the A2V mutation on proteasomal activity was measured in living human neuroblastoma SH-SY5Y cells stably transfected with human APP [24]. In cells expressing A673V-mutated APP, there was a higher increase in the release of Aβ peptides in the culture medium compared to cells with WT APP and an elevation in the C99:C88 ratio, indicating augmentation of the amyloidogenic pathway [24].

This observation was corroborated by a fluorescence resonance energy transfer-based assay performed in cell-free experiments employing a specific sequence for β-site APP-cleaving enzyme 1 (BACE1). Specifically, four peptides homologous to residues 668–675 of the APP in the wild-type form or carrying the Swedish, the A673T, or the A673V mutation were conjugated to the fluorogenic substrate 5-((2-aminoethyl) amino)naphthalene-1-sulfonic acid and the dark quencher molecules 4-((4-(dimethylamino)phenyl)azo)benzoic acid [24]. A similar BACE1 activity was determined for APP wild-type and that carrying A673T mutation. Notably, BACE1 activity was significantly inhibited for the substrate carrying the A673V substitution compared to the APP wild-type, A673T, and also that with the Swedish mutation [24].

These findings support the notion of interference with BACE1 activity by the presence of the A673V mutation on the APP gene, resulting in an augmentation of the amyloidogenic pathway. In addition, they indicate the effect on proteasomal activity by specific APP mutations and confirm an inverse relationship between Aβ level and proteasome activity, with significant implications for the pathogenesis of AD. The A673V mutation appears to interfere with proteasomal activity more than the other mutations, confirming the peptide's reduced degradation and potential for greater toxic action.

The effect of Aβ A2V mutation on cell toxicity was investigated by employing hippocampal neurons isolated from Brainbow mice, genetically engineered animals expressing fluorescent proteins in a stochastic and combinatorial manner, allowing for labeling and visualizing individual neurons with different colors [19]. For these experiments, neurons were exposed for 3 hours to oligomers of $A\beta1–42_{WT}$ or $A\beta1–42_{A2V}$ alone or obtained by an equimolar mix of the two peptides, and changes

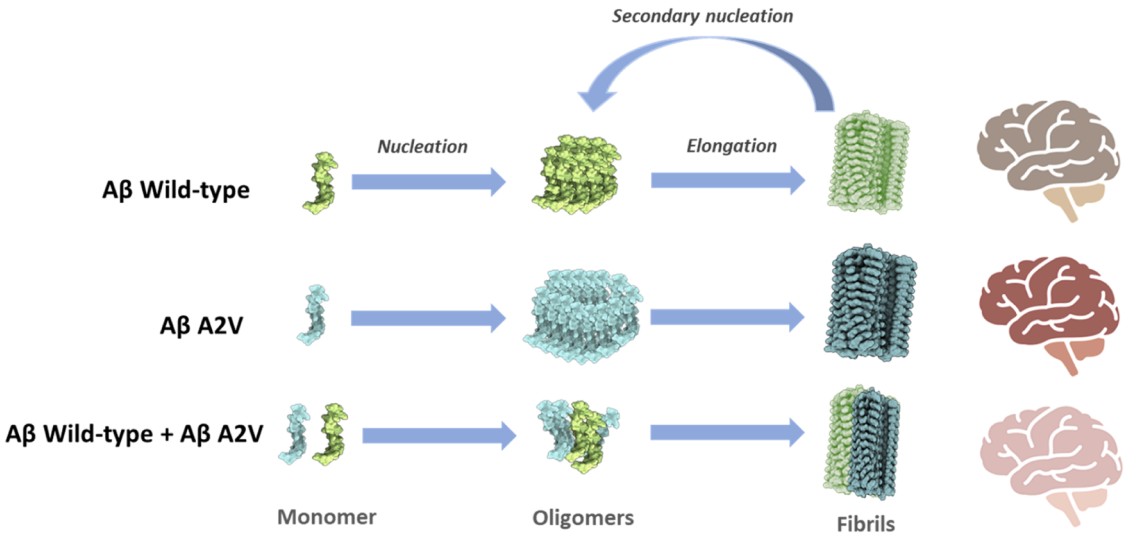

Fig. 2. Schematic representation of the impact of the AβA2V mutation in homozygous and heterozygous conditions on aggregation and toxicity. The A2V mutation in the homozygous condition promotes the adoption of a β-sheet structure by Aβ, increases primary oligomers, which are more toxic than those formed by Aβ WT, and promotes fibril production. A2V mutation also increases the dominance of secondary nucleation over primary nucleation and enhances the formation of amyloid aggregates with a more condensed structure. Conversely, in heterozygous conditions, the A2V mutation hinders the formation of toxic oligomers by disrupting stable intermolecular interactions with the wild-type sequence and reducing the aggregation. This translates into the formation of fibrils structurally different from those formed by A2V-mutated and wild-type Aβ. Created with BioRender.com.

in the biochemical composition of the postsynaptic density were evaluated. Additionally, time-lapse microscopy was utilized to analyze changes in the density of dendritic spines, the small protrusions on neurons that form synaptic connections with other neurons. This allowed the researchers to observe any alterations in synaptic structure induced by the different Aβ oligomers. Aβ1–42_{A2V} oligomers had a remarkable ability to trigger synaptic dysfunction and were more toxic than Aβ1–42_{WT} oligomers. Synaptic toxicity was observed in neurons at 1 μM Aβ1–42_{WT}, whereas Aβ1–42A2V was already toxic at 10 nM. Surprisingly, oligomers formed by mixing wild-type and A2V-mutated peptides did not induce synaptic toxicity, indicating that Aβ1–42_{A2V} can mitigate the effect of Aβ1–42_{WT} [19].

Different results were obtained when the *in vitro* synaptotoxicity of oligomeric wild-type and A2V- and A2T-mutated Aβ1–42 was evaluated by determining the inhibition of long term potentiation (LTP) in hippocampal cells [68]. Aβ1–42_{A2V} was as effective as wild-type in inhibiting LTP, suggesting that this mutation did not worsen the synaptic damage. On the other hand, the A2T-mutated Aβ1–42 was less effective than wild-type in inhibiting LTP [68].

To further investigate the toxic potential of oligomers formed by A2V-mutated Aβ, a surface plasmon resonance (SPR)-based immunoassay coupled with a behavioral test in the nematode *Caenorhabditis elegans* was performed [11,86]. The rhythmic contraction and relaxation of the *C. elegans* pharynx, known to be affected by various chemical stressors [47], is well known to be also influenced by biologically relevant oligomeric assemblies of amyloidogenic proteins [27,94]. These studies were performed using the Aβ1–40_{A2V} peptide based on a neuropathological analysis performed on the brain of the A673V homozygous proband, indicating that the deposition of Aβ1–40 species was overrepresented and that the effects of A2V genetic mutation might be more pronounced on Aβ1–40 than on Aβ1–42 [37]. Aβ1–40_{A2V} formed more oligomeric species than Aβ1–40_{WT}, as evaluated by surface plasmon resonance (SPR) analysis, which was recognized as toxic by *C. elegans* and caused a significant decrease in the pharyngeal pumping rate. These findings strongly indicated that A2V substitution drives the formation of more toxic oligomers (Fig. 2).

4.2. In Vivo Studies

Several attempts have been made to generate transgenic vertebrate animals expressing APP carrying A673V mutation but without success [24]. The only animal model still available to investigate whether A2V mutation affects Aβ assembly and toxicity *in vivo* is a transgenic *C. elegans* strain expressing at pan-neuronal level Aβ1–40_{WT} or Aβ1–40_{A2V} [27]. As the nematode lacks endogenous Aβ [33,58], expression of the mutated peptide reproduces the homozygous condition in humans. A transgenic *C. elegans* strain expressing Aβ1–40_{A2T} in neurons was also generated to elucidate the mechanisms underlying these substitutions' causative or protective effects, particularly on protein assembly and toxicity. Pan-neuronal expression of Aβ1–40_{A2T}, similar to what was observed with Aβ1–40_{WT} or Aβ1–40_{A2V} [27], was achieved by cloning the construct under the control of the *aex-3* neuronal promoter. The presence of A2V or A2T mutation increased the ability of Aβ to form soluble oligomeric assemblies *in vivo*. In particular, oligomeric assemblies slightly increased in A2T mutants and became significantly higher in worms expressing A2V mutated protein than in worms expressing Aβ1–40_{WT} [27]. These findings were confirmed by SPR studies with lysates from transgenic worms, which allowed a semiquantitative detection of oligomers passed over the 4G8 anti-Aβ antibody immobilized on a sensor chip. The 4G8-binding signal was higher for worms expressing A2V- and A2T-mutated Aβ1–40_{WT} than for those expressing Aβ1–40_{WT}. The shape of the sensorgrams also differed: most of the binding due to Aβ1–40_{WT} dissociated quickly, whereas most of the binding due to Aβ1–40_{A2V} did not, as expected for high-avidity binding due to the presence of oligomers. Moreover, A2T mutation promoted the propensity of Aβ1–40 to form toxic soluble assemblies *in vivo*, although at a lower extent than A2V-mutated peptide, confirming that a single amino acid substitution in the NT region results in pathologically relevant effects (Fig. 2).

The higher presence of oligomeric assemblies in the cytosols of nerve ring neurons of worms expressing Aβ1–40_{A2V}, compared with Aβ1–40_{WT} worms, was accompanied by morphological neuronal abnormalities, including swollen and enlarged mitochondria, behavioral abnormalities, and degenerative changes [27]. The expression of A2V-mutated Aβ also shortened worm survival by 44 % and median lifespan by 29 % compared to Aβ1–40_{WT}-expressing strains. The expression of A2V- or

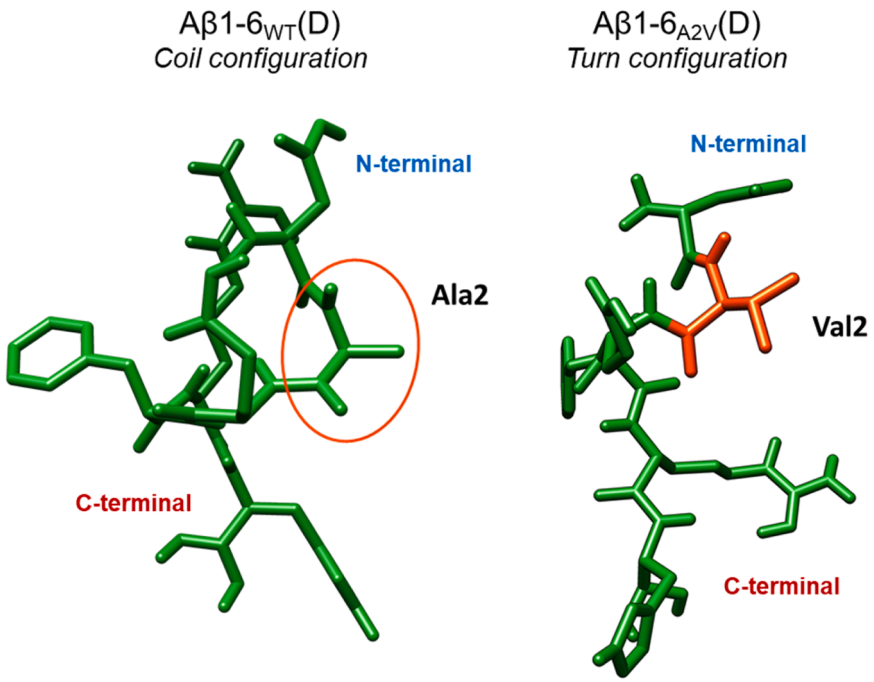

Fig. 3. Elongated "coil" configuration and compact "turn" configuration of Aβ1–6_{WT}(D) and Aβ1–6_{A2V}(D).

A2T-mutated Aβ1–40 caused a more significant impairment of the pharyngeal pumping than Aβ1–$40_{WT,}$ whereas the presence of A2V worsened the loss of motility caused by Aβ1–40_{WT} but not A2T substitution. The neuronal expression of mutated Aβ1–40 significantly compromised the postsynaptic compartments more than Aβ1–40_{WT}, which indicated that A2T and A2V transgenic worms were more resistant to aldicarb and levamisole than worms expressing Aβ1–40_{WT}.

5. TOWARD A NEW ANTI-AMYLOIDOGENIC PEPTIDE-BASED THERAPY

5.1. Design of a small Aβ peptide carrying the A2V mutation

The observation that A2V mutation affects the expression of defined AD-related targets inspired the design of a hexapeptide peptide based on the NT domain of Aβ carrying the A2V mutation, named Aβ1–6_{A2V}. This domain plays a crucial role in amyloidogenesis, and even a single amino acid change in its primary sequence affects assembly progression and aggregation kinetics [20,23,28]. Already in 2009, Di Fede et al. demonstrated that the Aβ1–6_{A2V} peptide binds more effectively than the Aβ1–6_{WT} peptide to Aβ1–40_{WT} fibrils and that the A2V-mutated Aβ1–6 inhibited the assembly of Aβ into amyloid fibrils [25]. These findings suggest that the heterotypic interaction between the mutated six-mer peptide and $Aβ_{WT}$ is favored by the amino acid substitution at position 2 and affects nucleation or nucleation-dependent Aβ polymerization, or both, hindering amyloidogenesis. These observations align with subsequent studies in which SPR analysis revealed that Aβ1–6_{A2V} significantly inhibits the elongation of Aβ1-42_{WT} amyloid fibrils by competing and ultimately antagonizing the docking and locking of Aβ monomers to the fibrils [24]. Circular dichroism spectroscopy showed that the heterotypic interaction between the mutated six-mer peptide and Aβ1–42_{WT} inhibited the acquisition of β-sheet secondary structure and was favored by the amino acid substitution at position 2. Electron microscopy and AFM studies demonstrated that Aβ1–6_{A2V} was more effective than Aβ1–6_{WT} in reducing the formation of Aβ1–40_{WT} protofibrils and filamentous structures [24].

The effect of Aβ1–6_{A2V} on the diffraction patterns of Aβ was investigated using the shortened CT Aβ1–28 sequence [14]. When the two peptides were co-incubated, they formed assemblies that appeared poorly oriented and nonfibrillar. Very weak X-ray diffraction and neutron scattering were observed for the mixture of Aβ1–28 and Aβ1–6_{A2V} at a 1:4 molar ratio with randomly distributed signals and low directionality, suggesting a lack of ordered structure. The amount of fibrils in this mixture was very low, and those present shared some characteristics with the fibrils formed by Aβ1–28 alone. Interestingly, the mixture did not exhibit a distinct meridional reflection, indicating a more significant hydrogen bonding direction disorder than the Aβ1–28 [14]. The absence of wide-angle diffraction in the mixture corresponded to the minimal detection of fibers in the small-angle profiles. Based on these findings, it was inferred that Aβ1–6_{A2V} disrupts or hinders interactions with Aβ1–28, suggesting a role for the mutated six-mer peptide in altering the structure and characteristics of the resulting assemblies.

These findings offered a proof of concept and established a foundation for the rational design of therapeutic agents based on Aβ1–6 fragments carrying the A2V mutation and peptidomimetic molecules that retain the fundamental functional properties of the mutated full-length Aβ for therapeutic purposes. Further steps in this strategy led to the development of a modified Aβ1–6_{A2V} formed only by D-amino acids [Aβ1–6_{A2V}(D)]. This peptide, similar to that formed by L-amino acids, can interact with full-length Aβ and obstruct the production of oligomers while preventing the formation of amyloid fibrils [24]. The use of amino acids in D isomeric form was also chosen to overcome problems of stability and the short half-life of the peptide in biological fluids [24].

SPR studies indicated that Aβ1–6_{A2V}(D) dose-dependently reduced the formation of Aβ1–42_{WT} oligomers [86], with an IC_{50} value of 80 μM and maximal effect at 300 μM. When Aβ1–6_{A2V}(D) was added to preformed oligomers, there was no observed effect, suggesting that the six-mer peptide interacted with soluble Aβ assemblies to prevent their formation but did not bind to preexisting Aβ oligomers [24].

Subsequently, a comparative conformation analysis of Aβ1–6_{WT} and Aβ1–6_{A2V} peptides was performed using classical MD simulations in explicit solvent to predict the structural basis of the anti-amyloidogenic effect of Aβ1–6_{A2V}(D). Both peptides were intrinsically disordered and flexible, but the substitution of Val-to-Ala in Aβ1–6_{A2V} altered the appearance of the peptide in solution, increasing the apolar character of

the solvent-accessible surface. Secondary structure content analysis showed that Aβ1–6_{A2V} and Aβ1–6_{WT} displayed a predominant coil configuration. Still, only Aβ1–6_{A2V} displayed a higher propensity to form secondary structure motifs involving two to three residues, particularly a turn involving the Glu3, Phe4, and Arg5 residues (Fig. 3). The propensity of the A2V mutant to adopt a Glu3-Arg5 turn configuration can explain its ability to hinder the assembly of full-length Aβ [23].

MD simulations showed that the anti-amyloidogenic activity of Aβ1–6_{A2V}(D) was associated with its structural flexibility (open-to-closed conformational flexibility), in contrast to the structural rigidity of Aβ1–6_{WT} (closed conformation) [23].

The observations obtained from a study of the Aβ1–6 peptides strongly correlate with the background information gathered from simulations of the Aβ1–40 and 1–42 peptides described in the previous sections. In particular, the mutation-induced change in partitioning between intra- and inter-molecular interactions discussed in the case of monomers and dimers of Aβ1–40 and 1–42 peptides supports this observation. Moreover, the work of Chakraborty and Das directly investigated the interactions between Aβ1–6 and the full peptide, uncovering the fact that mutated Aβ1–6 tends to interact more strongly with the full-length peptide compared with its WT counterpart [17]. This observation further supports the current understanding of the molecular basis of the inhibitory action of short peptides.

To improve the ability of Aβ1–6_{A2V}(D) to cross biological membranes, it was linked to a sequence homologous to residue 48–57 of TAT (GRKKRRQRRR), a short amino acid sequence derived from the HIV TAT peptide and` widely used for brain drug delivery [51]. The Aβ1–6_{A2V}TAT(D) peptide resisted degradation by endogenous proteases and remained stable when incubated for 48 hours in mouse serum [28]. Aβ1–6_{A2V}TAT(D), similarly to Aβ1–6_{A2V}(D), destabilized the secondary structure of Aβ1–42_{WT}, as evidenced by circular dichroism spectroscopy studies that showed inhibition of the acquisition of β-sheet conformation, leading to altered folding of the full-length peptide [23]. Aβ1–6_{A2V}TAT(D) prevented the ability of full-length Aβ to form fibrils *in vitro* by inhibiting the aggregation, as demonstrated by analysis using polarized light and electron microscopy [23]. However, Aβ1–6_{A2V}TAT(D) did not counteract the oligomerization of Aβ1–42, resulting in the formation of small globular structures and rare oligomeric structures [23].

5.2. Protective effect of the Aβ1–6_{A2V}(D) peptide against Aβ-induced toxicity

In vitro and *in vivo* studies were conducted to investigate the potential protective effect of Aβ1–6_{A2V}(D) against the toxicity caused by Aβ.

Both Aβ1–6_{WT}(D) and Aβ1–6_{A2V}(D) peptides, when tested on SH-SY5Y cells, showed no toxicity even at high concentrations (20 μM). Both could protect cells from the toxicity induced by Aβ1–42WT [23], although the mutated peptide exhibited a more substantial protective effect than Aβ1–6_{WT}(D). Additional experiments demonstrated that the Aβ1–6_{A2V}TAT(D) peptide, too, significantly protected SH-SY5Y cells, in a dose-dependent manner, from the toxicity induced by Aβ1–42_{WT} [24]. Aβ1–6_{A2V}TAT(D), at nanomolar concentrations, prevented the synaptic damage caused by 10 nM Aβ1–42_{WT} in hippocampal neuronal cells. In particular, the peptide protected cells from the reduction of synaptic protein levels, including GluN2A and GluN2B subunits of NMDA-receptor as well as in GluA1 and GluA2 subunits of AMPA-receptor and PSD-95 scaffold protein, and the loss of dendritic spines [19]. These findings indicate that Aβ1–6_{A2V}(D) and Aβ1–6_{A2V}TAT(D) peptides efficiently inhibit toxicity *in vitro*, positioning them as promising lead compounds for further studies assessing their potential in therapeutic intervention for AD.

The ability of Aβ1–6_{A2V}(D) peptide, whether linked to the TAT(D) sequence or not, to counteract the toxicity of Aβ1–42 oligomers was then assessed using *C. elegans,* able to recognize the proteotoxic forms of amyloidogenic proteins by reducing its pharyngeal function [86,93]. Both Aβ1–6_{A2V}(D) and Aβ1–6_{A2V}-TAT(D) peptides antagonized the pharyngeal impairment caused by oligomers, indicating that the protective activity of the A2V six-mer peptide was retained when it was linked to the TAT sequence.

Transgenic *C. elegans* strains expressing human Aβ in body wall muscle cells were then utilized to investigate the effectiveness of the Aβ1–6_{A2V}(D) and Aβ1–6_{A2V}TAT(D) peptides *in vivo* in mitigating Aβ polymerization and toxicity. Specifically, CL4176 transgenic worms, characterized by the accumulation of oligomeric Aβ1–42 assemblies [26], and CL2120 nematodes, which exhibit an age-related progressive reduction of motility associated with the accumulation of both Aβ fibrils and oligomers [79] were used. While these nematodes do not fully replicate the complexity of AD pathology, they serve as a complementary system valuable for screening and identifying small molecules affecting Aβ homeostasis and folding [5,6,64].

Aβ1–6_{A2V}TAT(D) but not Aβ1–6_{A2V}(D) protected CL4176 and CL2120 worms from the toxic phenotype, indicating that the TAT sequence, allowing translocation across biological membranes, is indispensable for the *in vivo* effect. Aβ1–6_{A2V}TAT(D) exerted a dose-dependent protective effect, with comparable IC_{50} values for both strains (47.4 ± 1.7 μM for CL4176 and 38.6 ± 1.2 μM for CL2120) [28]. The protective effect was associated with the capacity of the peptide to significantly impact Aβ oligomerization without interfering with *in vivo* Aβ production and degradation and with the ability of Aβ1–6_{A2V}TAT(D) to counteract dysfunction in nicotine-sensitive acetylcholinesterase receptors implicated in the motility defect of CL2120 worms [79].

The *in vivo* anti-amyloidogenic effect of Aβ1–6_{A2V}TAT(D) was also investigated in vertebrate AD models [19,23]. The safety and efficacy of the Aβ1–6_{A2V}TAT(D) peptide were first evaluated using TgCRND8 transgenic mice overexpressing human APP carrying Swedish and Indiana mutations [18]. This strain was employed as a known model of synaptopathy, cognitive deficits, impaired LTP, and spine loss [19]. For the *in vivo* toxicity study, 6-month-old TgCRND8 mice were given intraperitoneal injections of either TAT(D)-vehicle or a single or double injection of Aβ1–6_{A2V}TAT(D) peptide (20 mg/kg) every 2 weeks, and their weight was monitored continuously. For the *in vivo* neuroprotection study, 6-month-old TgCRND8 mice were treated with either TAT(D)-vehicle or a single intraperitoneal injection of Aβ1–6_{A2V}TAT(D) peptide (20 mg/kg) [19]. Monitoring of animal weight indicated no significant differences among the groups, suggesting no apparent toxicity of the peptide after 1 month of administration [19]. The Aβ1–6_{A2V}TAT(D) peptide crossed the blood-brain barrier, reaching the brain within 6 hours after administration, and distributed in the liver and kidneys, with notable brain retention even 15 days after treatment [19]. Subsequent tests evaluated the neuroprotective effect of the peptide against spine injury. TgCRND8 mice treated with Aβ1–6_{A2V}TAT(D) displayed improvements in postsynaptic density composition compared to control mice, indicated by increased levels of GluN2A, GluA1, GluA2 receptors, and PSD-95 scaffold protein. These findings suggest that the peptide partially mitigates AD synaptopathy in TgCRND8 mice, highlighting its potential as a therapeutic intervention against AD-related synaptic dysfunction.

An *in vivo* study was then conducted on transgenic APP23 mice carrying a hybrid mouse/human APP gene with the Swedish mutation and a human PS1 gene with the dE9 mutation (APPswe/PS1dE9) to prevent interference from murine APP in the peptide's anti-amyloidogenic properties [23]. Aβ1–6_{A2V}TAT(D) was intraperitoneally administered once a week at 10 mg/kg body weight (b.w.) to APPswe/PS1dE9 mice 4 months of age when amyloid deposition typically starts. Animals were subjected to behavioral studies and biological evaluations 2.5 and 5 months after the start of treatment. The 2.5-month treatment resulted in a significant decrease in Aβ production and aggregation and the prevention of amyloid deposition in the brain. Aβ1–6_{A2V}TAT(D) increased the Aβ1–42 soluble fraction and decreased the insoluble fraction, suggesting a reduction of the amount of Aβ1–42 prone to deposit in the brain. This finding was supported by reduced

Table 1
Effects of Aβ1–6_{A2V}(D) peptide on *in vitro* and *in vivo* Aβ toxicity.

Effect	Model system	Treatment	Outcomes & Measurements	Reference
Protection against Aβ1–42_{WT}-induced toxicity	SH-SY5Y cells	Aβ1–6_{A2V}(D) and Aβ1–6_{A2V}TAT(D) peptides tested against Aβ1–42_{WT} toxicity.	Cell viability assays, synaptic protein levels (GluN2A/B, GluA1/A2, PSD−95), dendritic spine density.	[24], [23] [19]
Protection from oligomeric Aβ1–42_{WT} toxicity	*C. elegans* expressing human Aβ	Aβ1–6_{A2V}(D) and Aβ1–6_{A2V}TAT(D) peptides oligomeric and fibrillar Aβ1–42_{WT} toxicity	Pharyngeal function, motility impairment, Aβ polymerization, and toxicity.	[86] [23]
Protection against synaptic dysfunction	TGCRND8 mice	Intraperitoneal injection of Aβ1–6_{A2V}TAT (D) (20 mg/kg b.w. every 2 weeks for 1 month)	Weight monitoring, synaptic density (GluN2A, GluA1/A2, PSD−95), brain biodistribution of peptide.	[19]
Reduction of Aβ oligomers formation and amyloid deposition	APPSWE/PS1DE9	Intranasal administration of Aβ1–6_{A2V}(D) (50 mg/kg b.w. every 48 hours for 20 weeks)	Aβ aggregation, Aβ levels (soluble/insoluble fractions), APP processing (C99/C83), amyloid burden (immunohistochemistry).	[15]
Improvement of cognitive performance	APPSWE/PS1DE9	Novel object recognition test after intranasal treatment for 2.5 and 5 months with Aβ1–6_{A2V}TAT(D)	Cognitive performance, Aβ aggregation, amyloid deposition.	[23]
Prevention of Aβ aggregation and amyloid deposition	APPSWE/PS1DE9	Intranasal administration of Aβ1–6_{A2V}(D) (50 mg/kg b.w. every 48 hours for 20 weeks)	Aβ aggregation, Aβ levels (soluble/insoluble fractions), APP processing (C99/C83), amyloid burden (immunohistochemistry).	[15]
Reduced amyloid burden	3xTg-AD mice + TBI	Intranasal administration of Aβ1–6_{A2V}(D) (50 mg/kg b.w.)	Biochemical analysis of Aβ levels.	[29]

amyloid deposition in all brain regions, including the hippocampus, and was most pronounced in the frontal and entorhinal cortices and olfactory bulbs [23].

Furthermore, the reduction of aggregated Aβ was associated with an increase in monomeric Aβ1–42 levels, indicating that Aβ1–6_{A2V}TAT(D)-based therapy changes the equilibrium between Aβ aggregates and monomers, leading to a reduction of the most toxic Aβ species. Aβ1–6_{A2V}TAT(D) treatment positively affected the mice's cognitive function, as evidenced by their novel object recognition test (NOR) performance. These findings were consistent with the observed reduction in Aβ production and aggregation and preventing amyloid deposition in the brain [23]. However, after 5 months of treatment, the study's results were unexpected, with an effect of the peptide on Aβ production lower than that observed after 2.5 months and a substantial increase in an amyloid buildup. Nevertheless, the NOR showed preserved cognitive function in the Aβ1–6_{A2V}TAT(D) group compared to the control group, with significant differences in recognition and discrimination scores [23]. In conclusion, even though the short-term treatment prevented cognitive deterioration, Aβ aggregation, and amyloid deposition in the brain, the extended treatment schedule, while retaining the results for cognitive impairment, attenuated the effects on Aβ production and increased amyloid burden. This was subsequently attributed to the amyloidogenic nature of the TAT carrier [38], which interfered with the anti-amyloidogenic properties of Aβ1–6_{A2V}(D).

To allow the peptide to reach the brain without TAT, intranasal administration of Aβ1–6_{A2V}(D) administration was developed to avoid potential side effects or counteractions caused by the carrier [15]. Biodistribution studies were first performed on transgenic APPSwe/PS1dE9 mice treated with 50 mg/kg b.w. Aβ1–6_{A2V}(D). As an assisted laser desorption ionization – time of flight imaging study demonstrated, intranasal administration allows distribution in various brain regions, including the cerebral cortex, hippocampus, caudate-putamen, and cerebellum. The peptide was still observed in the cerebral cortex 48 hours after the last treatment. Peptide quantification indicated that the highest concentration was observed in the cortex and hippocampus 4 hours after treatment. There was a slight decrease in peptide levels at 24 hours, but in the striatum, the highest level of the peptide was observed at 4 and 24 hours and decreased at 48 hours [15]. Based on these findings, a treatment schedule with a 48-hour interval between intranasal administrations of 50 mg/kg b.w. Aβ1–6_{A2V}(D) for 20 weeks was chosen to determine its efficacy. The impact of Aβ1–6_{A2V}(D) on the aggregation and production of Aβ was evaluated at the end of the treatment. Although the total levels of Aβ40 and Aβ42 did not differ between brain homogenates from mice treated with Aβ1–6_{A2V}(D) and the control group, there was a significant decrease in oligomeric Aβ levels in both soluble and insoluble fractions, indicating that the peptide can inhibit the formation of toxic Aβ assemblies *in vivo*. The extended treatment with Aβ1–6_{A2V}(D) also had little effect on APP processing [15]. In particular, APP processing involves enzymatic cleavage, yielding amyloidogenic fragments like C99 and non-amyloidogenic fragments like C83. The levels of these fragments were analyzed using a western blot with the A8717 antibody. From the results of the densitometric analysis, no significant difference was observed in the levels of C99 and C83 between mice treated with the peptide and untreated controls. Consequently, the extended treatment with Aβ1–6_{A2V}(D) did not significantly alter the processing of APP, indicating its limited effect in this regard [15].

Immunohistochemical studies also indicated that the administration of Aβ1–6_{A2V}(D) effectively prevented the formation of Aβ deposits in the brains of mice. This effect was observed even in brain regions distant from the olfactory bulbs, which were actively involved in forming amyloid plaques in the APPSwe/PS1dE9 model. These findings were validated by densitometric analysis of the amyloid burden, which demonstrated a significant decrease in Aβ deposition in the brain of mice treated with Aβ1–6_{A2V}(D) [15].

The results obtained from these studies, summarized in Table 1, indicate that Aβ1–6_{A2V}(D) prevented Aβ oligomerization, deposition, and synaptic damage in the mouse model of AD.

5.3. *Protective effect of the Aβ1–6_{A2V}(D) peptide against tau-induced toxicity*

A comprehensive biochemical analysis was recently conducted to assess Aβ1–6_{A2V}(D)'s ability to interfere with the aggregation of tau, its stability, and its toxicity [29]. A multidisciplinary approach was employed for this study, involving biophysical and biochemical analysis on recombinant tau and tau extracted from the brains of transgenic mice modeling tauopathy. Experiments were also done using *C. elegans* to recognize the toxic tau assemblies produced *in vitro* and *in vivo*. Lastly, animal models predisposed to AD or exposed to traumatic brain injury (TBI), a known AD risk factor [93], were used.

The potential of the Aβ1–6_{A2V}(D) peptide in countering tau aggregation was investigated by thioflavin-T (ThT) fluorescence assay and AFM analysis using recombinant tau in the WT and P301L-mutated form. The peptide effectively reduced the aggregation propensity of Tau WT and P301L. To investigate whether the peptide can also reduce the formation of tau proteotoxic assemblies, brain homogenates from P301L transgenic mice, which served as a source of misfolded toxic tau,

Table 2
Effects of Aβ1–6_{A2V}(D) peptide on tau toxicity.

Effect	Model System	Treatment	Outcomes & Measurements	Reference
Protection from oligomeric tau toxicity	*C. elegans*	Brain homogenates from P301L transgenic mice incubated with Aβ1–6_{A2V}(D) or Aβ1–6_{A2V}TAT(D)	IC_{50} value, locomotor toxicity measurements.	[93] [29]
Reduced oligomeric tau toxicity and memory improvement after TBI	3xTg-AD mice + TBI	Intranasal administration of Aβ1–6_{A2V}(D) (50 mg/kg b.w.) every 48 hours.	Y-maze spatial memory test. Locomotor toxicity of brain homogenates to *C. elegans*.	[29]
Reduced phosphorylated tau, and P-tau/tau ratio, improved locomotor activity and reduced axonal damage	Aged female mice + TBI	Intranasal administration of Aβ1–6_{A2V}(D) (50 mg/kg b.w.).	Biochemical analysis of phosphorylated tau and total tau. Locomotor activity, sensorimotor tests, and plasma neurofilament light levels.	[29]

were incubated with Aβ1–6_{A2V}TAT(D) or Aβ1–6_{A2V}(D) before administration to *C. elegans*. Only the Aβ1–6_{A2V}TAT(D) peptide protected worms from toxicity, with an IC_{50} value of 25.60 μM, demonstrating that, similarly to what was observed with Aβ-induced toxicity, also for tau, the TAT sequence is indispensable for mediating the efficient passage and cellular uptake of the peptide within the cells of *C. elegans*. Aβ1–6_{A2V}TAT(D) also counteracted the toxicity caused by the administration to worms of misfolded and hyperphosphorylated tau generated in wild-type mice 12 months post-TBI [93]. Biochemical analysis revealed that incubation of brain homogenates from P301L transgenic mice with Aβ1–6_{A2V}TAT(D) or Aβ1–6_{A2V}(D), but not TAT alone, significantly reduced tau protein levels without affecting the fraction of insoluble tau. This was attributed to the peptide's ability to increase tau's susceptibility to protease degradation, as was particularly evident in the presence of proteinase K.

Subsequently, the *in vivo* efficacy of Aβ1–6_{A2V}(D) was assessed in young 3xTg-AD mice used as a model of AD associated with plaque and tangle pathology with synaptic dysfunction, subjected to TBI to accelerate tau deposition. The intranasal administration of mice treated with 50 mg/kg b.w. Aβ1–6_{A2V}(D) resulted in an efficient distribution of the peptide in the brain of TBI mice 4 hours after treatment, which persisted for up to 48 hours. Based on this, a treatment schedule involving intranasal administrations every 48 hours was chosen. The treatment of 3xTg-AD mice with Aβ1–6_{A2V}(D) before TBI significantly attenuated memory impairment, as demonstrated by improved performance in the Y-maze spatial memory test [29].

Additionally, brain homogenates from 3xTg-AD -TBI mice treated with Aβ1–6_{A2V}(D) were significantly less toxic to *C. elegans* than untreated ones, proving the ability of the peptide to reduce the formation of proteotoxic species also *in vivo*. Biochemical analysis performed on brain homogenates of TBI mice demonstrated that although Aβ1–6_{A2V}(D) did not significantly affect the total levels of human tau or phosphorylated tau, it notably reduced Aβ levels, indicating a reduction in amyloid burden. This effect may be partly attributed to the peptide's ability to inhibit BACE activity [29].

Lastly, the protective effect of Aβ1–6_{A2V}(D) was investigated in aged female mice subjected to TBI, modeling the high risk of accidental falls among elderly individuals, particularly elderly women. In line with human aging, aging mice exhibit increased tau deposition in the brain, particularly in females, who show higher tau pathology correlated with neurological dysfunction [29]. Unlike humans, aged mice do not develop Aβ plaques, making them suitable for testing whether Aβ1–6_{A2V}(D) protection persists without Aβ pathology. TBI mice intranasally treated with 50 mg/kg b.w. Aβ1–6_{A2V}(D) showed significantly improved sensorimotor impairments 7 days post-injury.

Similarly, Aβ1–6_{A2V}(D) treatment prevented the reduction in locomotor activity, ameliorated the memory deficits, and reduced plasmatic neurofilament light levels, indicating protection against axonal damage caused by TBI. Biochemical analysis of brain homogenates revealed that Aβ1–6_{A2V}(D) significantly reduced phosphorylated tau levels and the P-tau/tau ratio. Moreover, brain homogenates from Aβ1–6_{A2V}(D)-treated mice did not induce locomotor deficits in *C. elegans,* indicating the ability of the peptide to reduce the formation of tau proteotoxic species *in vivo* [29].

Overall, these findings demonstrate the peculiar ability of Aβ1–6_{A2V}(D) to interact not only with Aβ but also with tau, hindering its aggregation and toxicity, both *in vitro* and *in vivo* (Table 2). This makes it a promising candidate for therapeutic development against AD.

6. CONCLUSIONS

For several years, extensive research has been conducted on disease-modifying treatments for AD. In 2020, clinical trials evaluated at least 121 agents, and numerous others are currently being studied in preclinical research [21]. Numerous drug candidates have been proposed in recent years, focusing on non-amyloid targets, such as with anti-inflammatory drugs, anti-tau drugs, and compounds targeting synapses, vascular factors, and neurogenesis. Recently, the U.S. Food and Drug Administration approved lecanemab, a humanized monoclonal antibody that binds with high affinity to Aβ soluble protofibrils, for treating AD patients with mild cognitive impairment or mild dementia [54]. This is the second antibody approved for this disease after aducanumab [9]. The benefits associated with using this class of drugs remain to be established. They are costly and require continuous monitoring of patients, thus implying a considerable expenditure of resources for the healthcare system. Furthermore, there is still significant debate on the ability of these antibodies, particularly aducanumab, to induce small cerebral hemorrhages and amyloid-related imaging abnormalities associated with cerebral edema and neurological disorders [81]. Despite these efforts, all approaches have so far failed to effectively stop the onset and progression of the disease.

The failure of therapeutic strategies against AD may be attributed to multiple reasons, including the weak rationality of some approaches based on theoretical assumptions insufficient validation in animal models, which impedes their translation to clinical practice [46,69]; inadequate timing of therapeutic intervention [71]; the phenotypic variability of AD, which impacts the response to treatments; and the complexity of the disease's pathogenesis. This suggests that therapeutic strategies should address multiple targets, which are likely among the various causes for the failure of therapeutic strategies against AD, to effectively combat the illness [32,36,65]. Findings imply that the best approach for treating AD would involve multiple interrelated molecular mechanisms by disrupting the well-known factors involved in AD pathogenesis (such as Aβ, tau, and neuroinflammation) and blocking their neurotoxic effects.

Genetic research in the recent past has identified uncommon mutations that potentially offer protection against AD, providing a promising foundation for developing new investigational compounds to treat the disease [14,48,63,75]. Taking inspiration from genetics, a potential strategy for developing an effective treatment for AD is to explore the protective effects of genetic variants found in nature. Based on this idea, the Istituto di Richerche Farmacologiche Mario Negri and the Istituto Neurologico Carlo Besta developed a new compound able to mimic the protective properties of the $Aβ_{A2V}$ variant, which occurs naturally in

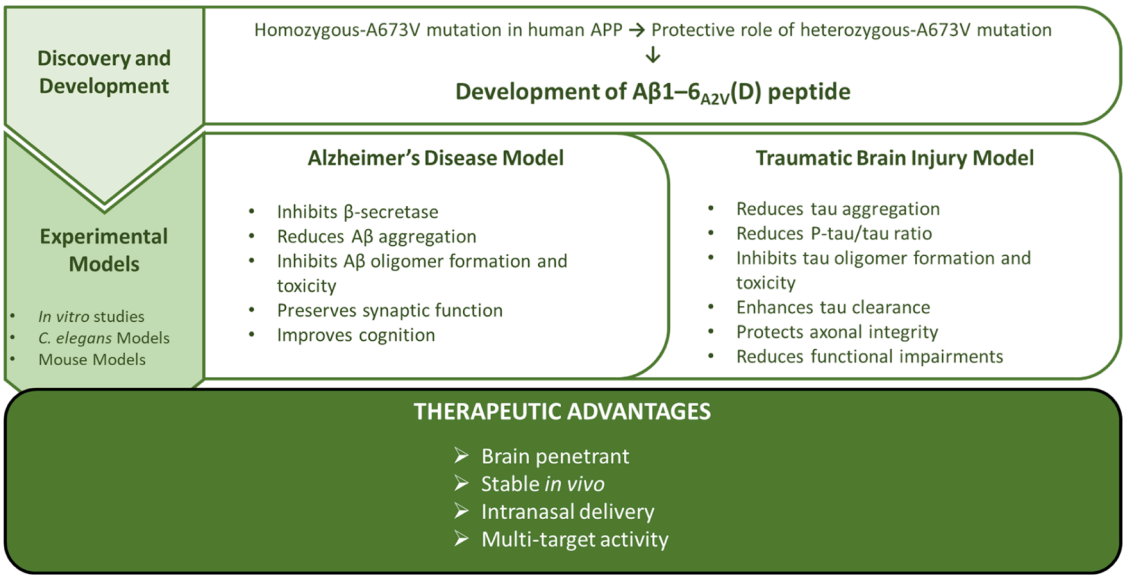

Fig. 4. AβA2V Paradigm: A Multi-Target Therapeutic Strategy. The figure illustrates the discovery and development of the Aβ1–6_{A2V}(D) peptide, its evaluation in experimental models, and its therapeutic potential in Alzheimer's disease and traumatic brain injury as models of tauopathies. The mechanisms of action and therapeutic advantages are highlighted, showing its broad applicability in targeting multiple pathological pathways.

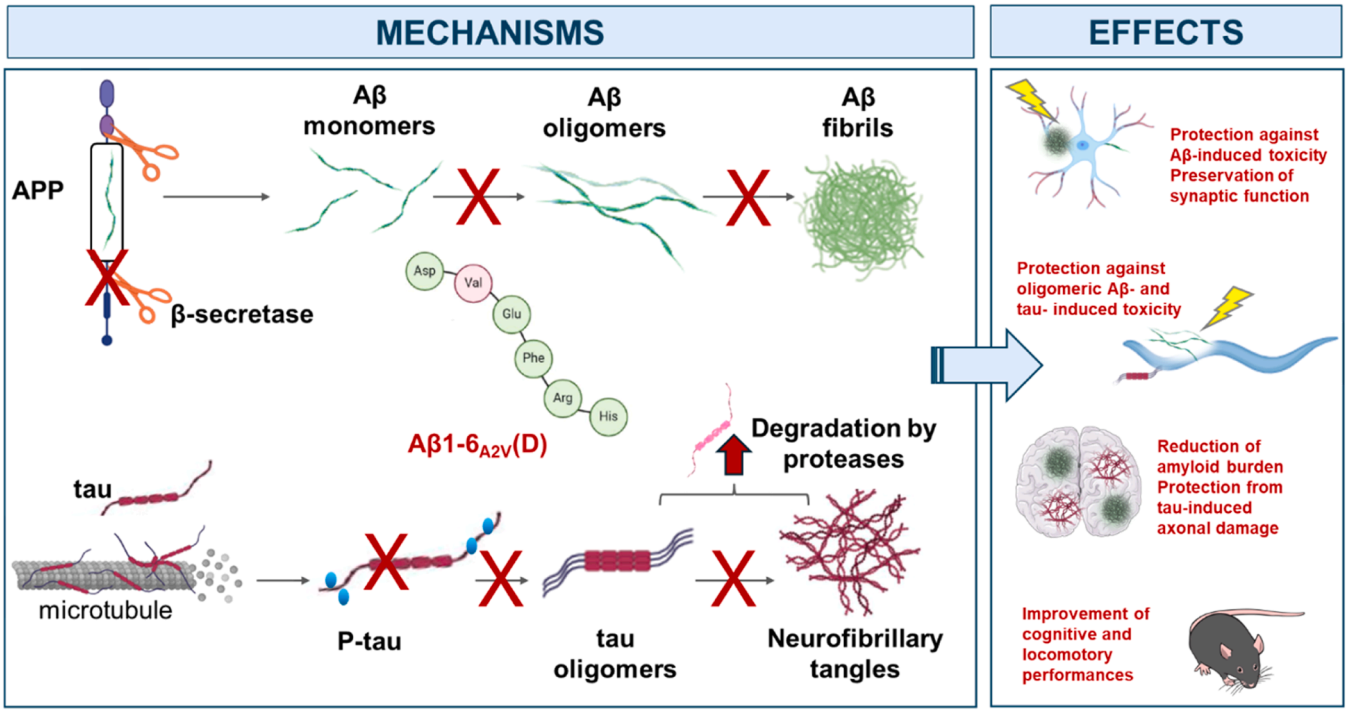

Fig. 5. Summary of the mechanisms and effects exerted by Aβ1–6_{A2V}(D) on Aβ and Tau. The Aβ1–6_{A2V}(D) peptide can exert different mechanisms on Aβ and tau. It can inhibit the activity of the β-secretase, preventing the generation of Aβ from amyloid precursor protein (APP). Furthermore, Aβ1–6_{A2V}(D) inhibits aggregation of the Aβ monomeric peptide into oligomeric forms, limiting the formation and deposition of amyloid aggregates. Additionally, Aβ1–6_{A2V}(D) reduced the phosphorylation of the tau protein released in pathologic conditions from microtubules, decreasing the ratio between phosphorylated and non-phosphorylated tau. This effect is also accompanied by a reduction of the formation of tau oligomers and by an increased susceptibility of misfolded tau to endogenous proteases. All these effects result in a decrease in the formation and deposition of neurofibrillary tangles. Thanks to its multitarget activity, Aβ1–6_{A2V}(D) exerts protective effects against both Aβ and tau toxicities, proven in vitro and in vivo models, reduces the amyloid burden and improves the cognitive and locomotory performances in transgenic mice modeling Alzheimer's disease. Created with BioRender.com.

humans with a lower risk of developing AD [24,25,28,29].

According to the evidence presented here and summarized in Tables 1 and 2, it is believed that Aβ1–6_{A2V}(D) could be a promising treatment for preventing amyloidogenesis and its harmful effects on cognitive function and synaptic activity in AD. The use of D-peptides is auspicious because of their stability, bioavailability [69], and strong resistance to protease degradation, making them excellent candidates for developing drugs to treat neurological disorders [52]. In fact, Aβ1–6_{A2V}(D) could be categorized as a type of "amyloid β-targeted peptide inhibitor" [15] that possesses unique characteristics such as

high specificity, low tissue accumulation, minimal adverse effects and toxicity, and various chemical and biological synthesis methods that distinguish them from other drugs used in therapy [39,44,46].

To conclude this review, we could sum up the $A\beta_{A2V}$-based approach as possessing two key advantages over previous AD therapies [16,59,90] (Figs. 4 and 5). First, it is based on a naturally existing "protective" model seen in APP-A673V heterozygous carriers who are shielded from AD. Second, it employs a combination of mechanisms that produce multiple beneficial effects on AD and primary tauopathies pathogenesis, including hindering oligomer production, fibril formation, and amyloid accumulation, as well as interrupting Aβ- and tau-associated neurotoxicity, and synaptic dysfunction. These actions have been shown to delay cognitive decline in animal models. The use of $A\beta1–6_{A2V}(D)$ as a therapeutic strategy for AD is advantageous in several ways: not only is it less expensive compared to other pharmacological treatments like monoclonal antibodies, but also, thanks to the intranasal administration, it should ensure high compliance among AD patients. Most importantly, a preclinical study on APPSwe/PS1dE9 mice showed that early administration of $A\beta1–6_{A2V}(D)$ effectively prevented amyloid deposits in the brain [15], reduced the amyloid burden and protected from the TBI-induced functional impairment [29]. This suggests that the bio-inspired $A\beta_{A2V}$-based strategy can be used as a preventive or curative approach to AD and primary tauopathies. However, further studies are needed to determine the most effective treatment schedule and to evaluate the potential side effects of $A\beta1–6_{A2V}(D)$, which have not yet been observed in preclinical studies.

These promising results warrant further investigation and clinical trials to determine the safety and efficacy of this therapy in human patients with AD. Overall, the discovery of the A2V mutation and preclinical validation of the $A\beta1–6_{A2V}(D)$ peptide therapy provide hope for developing effective treatments for AD and underscore the importance of continued research in this field to satisfy the need for innovative therapeutic approaches.

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CRediT authorship contribution statement

Andrea Conz: Writing – review & editing, Writing – original draft. **Michele Mosconi:** Writing – review & editing. **Mario Salmona:** Supervision. **Luisa Diomede:** Supervision. **Giuseppe Di Fede:** Writing – review & editing, Writing – original draft, Conceptualization. **Fabrizio Tagliavini:** Writing – review & editing, Writing – original draft, Conceptualization. **Laura Colombo:** Investigation. **Marcella Catania:** Writing – original draft, Conceptualization. **Matteo Salvalaglio:** Writing – review & editing, Writing – original draft. **Alfredo Cagnotto:** Writing – original draft, Investigation. **Tatiana Stoilova:** Writing – original draft. **Matteo Paloni:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authorship Contributions

Participated in review design: Diomede L., Salmona M., Di Fede G., Catania M., Tagliavini F. *Wrote or contributed to the writing of the manuscript*: Diomede L., Salmona M., Conz A., Mosconi M., Salvalaglio M., Stoilova T, Paloni M. *Revised the manuscript*: Di Fede G., Catania M., Tagliavini F., Colombo L., Cagnotto A.

Data Availability

This is a review based on data available in literature.

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