APP/Aβ Structural Diversity and Alzheimer's Disease Pathogenesis

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Abstract

The amyloid cascade hypothesis of Alzheimer's disease (AD) proposes amyloid- β (Aβ) is a chief pathological element of dementia. AD therapies have targeted monomeric and oligomeric Aβ peptides. However, alternative APP proteolytic processing produces a complex roster of Aβ species. In addition, Aβ peptides are subject to extensive posttranslational modification (PTM). We propose that amplified production of some APP/Aβ species, perhaps exacerbated by differential gene expression and reduced peptide degradation, creates a diverse spectrum of modified species which disrupt brain homeostasis and accelerate AD neurodegeneration. We surveyed the literature to catalog Aβ PTM including species with isoAsp at positions 7 and 23 which may phenocopy the Tottori and Iowa Aβ mutations that result in early onset AD. We speculate that accumulation of these alterations induce drastic changes in secondary and tertiary structure of Aβ that favor increased toxicity, and seeding and propagation in sporadic AD. Additionally, amyloid-β peptides with a pyroglutamate modification at position 3 and oxidation of Met35 make up a substantial portion of sporadic AD amyloid deposits. The intrinsic physical properties of these species including resistance to degradation, fast aggregation rate, increased neurotoxicity, and association with behavioral deficits suggest their emergence are linked to dementia. The generation of specific 3D-molecular conformations of Aβ impart unique biophysical properties and a capacity to seed the prion-like global transmission of amyloid through the brain. The relentless accumulation of rogue Aβ ultimately contributes to the destruction of vascular walls, neurons and glial cells culminating in dementia. A systematic examination of Aβ PTM and a probing correlation of their toxicity may help create essential biomarkers to

more precisely stage AD pathology, design countermeasures and gauge the impacts of interventions.

Introduction

Alzheimer's disease (AD) is characterized by the deposition of amyloid plaques and neurofibrillary tangles (NFT) in the brain. The main component of extracellular amyloid plaques is the amyloid- β peptide (Aβ), an approximately 4 kDa fragment derived from the larger amyloid precursor protein (APP) by the concerted action of β- and ɣ-secretases **[1]**. The Aβ peptides polymerize into insoluble ~10 nm filaments which accumulate in senile plaques and the walls of cerebral blood vessels. The NFT are aberrant aggregates mainly composed of tau, a phosphorylated microtubule-associated protein that aggregates into insoluble intraneuronal paired helical filaments **[2].** While recognizing the importance of NFT as potential co-pathogenic species in AD, in this critical review we focus specifically on the role of Aβ.

The evolutionary conservation of Aβ suggests this molecule has an adaptive value and important function(s) in the maintenance of CNS homeostasis. Of all 30 mammalian orders, which began to diverge about 90 million years ago, rodents are the only known species harboring amino acid substitutions deviating from the ancestral Aβ sequence. In sharp contrast with humans and many other mammals, **with the exception of the brush tailed rat** (Roychaudhuri R. Zheng X. Lomakin A. Maiti P. Condron MM. Benedek GB. Bitan G. Bowers MT. and Teplow DB. Role of species-specific primary structure differences in Aβ42 assembly and neurotoxicity. ACS Chem. Neurosci. 2015, 6, 1941-55), age-associated amyloid deposits do not accumulate in rodents *in vivo* **[3]**, even though synthetic rodent Aβ peptides produce congophilic filaments *in vitro* **[4,5].** Animal and cellular models are necessary for ascertaining disease mechanisms and promoting drug discovery efforts. However, there are still considerable challenges in translating scientific findings from these models into effective clinical interventions.

The amyloid cascade hypothesis is currently the most widely accepted general theory to explain the pathophysiology and clinical evolution of AD. The hypothesis posits Aβ40 and Aβ42 peptides are the critical elements in AD pathogenesis, through their intra- or extracellular neuropil and vascular accumulation. Notwithstanding the genetic evidence suggesting a crucial role for Aβ, considerable controversy still exists over the precise role(s) of amyloid in AD pathogenesis and pathophysiology **[6-8]**. Amyloid plaques correlate weakly with the clinical progression of AD and are preceded by tau neurodegeneration and brain atrophy in limbic brain regions **[9-18]**. To account for discrepancies between amyloid deposition and AD dementia some investigators suggest that soluble oligomeric Aβ are the most toxic species. The literature pertaining to the role of oligomeric Aβ in the pathogenesis and pathophysiology of AD is extensive **[19-22]** with almost 5,000 articles listed under "oligomeric A-beta" in PubMed. Excellent reviews on these topics can be found in references **[1,22,25-28]** However, no consensus exists regarding the molecular form(s) of Aβ ultimately responsible for the neurological decline associated with AD, the form(s) which should be therapeutically targeted or the optimal time to commence treatment. The timing of the initial Aβ accumulation and its propagation during the course of disease remains controversial

[23]. Likewise, whether Aβ accumulation in the CNS is influenced by Aβ pools originating from peripheral tissues and/or the systemic circulation is unclear **[24-27]**.

The hallmark of AD amyloid found in demented subjects is its immense complexity. Commonly presumed to be composed of Aβ40 and Aβ42 species, extensive posttranslational modifications (PTM) produce a wide array of molecules differing in physical size and chemical/conformation properties. Analogous to the situation observed with other proteinopathies, some of these potentially toxic modified Aβ conformers may promote the proliferation of highly organized amyloid filaments **[28-30].**

We hypothesize that in late onset AD (LOAD), specific Aβ-related species with shorter or longer sequences and /or altered by PTM enhance noxious amyloid deposition and neurotoxicity. Based on these assumptions, we review experimental evidence revealing the physicochemical nature of potentially neurotoxic amyloid species linked to AD. We consider neglected factors such as covalent modifications of Aβ and its aggregation states that may influence AD pathophysiology and have important implications for the design of immunotherapies. We consider APP proteolysis fragments and peripheral Aβ sources as potential factors influencing neurodegeneration and cognitive dysfunction. In addition, we propose tactics to aid the search for prospective Aβ biomarkers and therapeutic targets.

Amyloid-β posttranslational modifications and AD pathophysiology.

Structural alterations in the peptide backbone of Aβ could account for the differential deposition and stability of these molecules in AD **[31].** Detailed analyses have revealed that the species present in AD brains are modified extensively **[32]**. Furthermore, the Aβ peptides isolated from amyloid plaque cores possess a heterogeneous array of N- and C-termini and variable quantities of water soluble and water insoluble Aβ **[31,33]**. The fundamental chemical characteristics of the Aβ polypeptides are dictated by the amphipathic nature of these molecules, the presence of non-polar and polar domains and an abundance of charged amino acid residues which impose a diverse array of secondary and tertiary structures. Amyloid-β peptides ending in residues 38 to 49, a part of the transmembrane domain of the APP molecule, are progressively more hydrophobic due to the enrichment of non-polar amino acids which decrease solubility and increase aggregation propensity. The removal of charged amino acid residues at the N-terminal region of Aβ by aminopeptidases, endopeptidases or modification by glutaminyl cyclase will also have critical consequences for the intermolecular ionic interactions of the Aβ peptides since this region contains Asp and Glu at positions 1, 3, 7 and 11, and Arg, Lys and His at positions 5, 6, 13, 14 and 16. Deletions or additions in the Aβ sequence will result in differences in molecular folding patterns and intermolecular reactivity. The central domain of Aβ from Leu17 to Lys28 also contains a conserved hydrophobic domain (Leu17-Val18-Phe19-Phe20-Ala) and the negatively charged residues Glu22 and Asp23. In the following section we give an account of the most important PTM present in the Aβ peptides.

Aspartyl isomerization

Aspartic acid and asparagine residues are particularly subject to non-enzymatic degradation reactions that covalently alter the structure of the polypeptide chain.

The proximity of the side chain carbonyl group of Asp/Asn to the adjacent residue amide nitrogen induces the formation of a five-membered succinimide ring intermediate **[34]** which is subject to enhanced racemization **[35]**. Spontaneous hydrolysis of the L- and D-succinimide intermediates generate a mixture of L- and D-aspartyl and L- and D-isoaspartyl residues **[34]**. The presence of the isoaspartyl residue distorts the peptide chain to give a kinked polypeptide conformation that resembles a C-terminal substituted Asn residue. Racemization may also occur via radical reactions **[36]**. L-isoaspartyl residues (and to a lesser extent D-aspartyl residues) can be recognized intracellularly by the protein L-IsoAspartyl (D-aspartyl) O-methyltransferase (PIMT) which converts them to L-aspartyl and D-isoaspartyl residues **[37]**. Tryptic digestion and reverse-phase HPLC separation of AD Aβ peptides yielded several isoforms comprising residues Aβ1-5 and Aβ6-16 **[31]**. Amino acid composition, amino acid sequence analysis, mass spectrometry, enzymatic methylation and stereoisomer determinations demonstrated structural rearrangements of Asp residues at positions Aβ1 and Aβ7. L-isoAsp was the predominant form with D-isoAsp, L-Asp and D-Asp present as minor components, as would be expected for succinimide-mediated degradation. Approximately 75% of the Aβ peptides in the AD brain parenchymal amyloid plaque cores contain isoAsp at position Aβ7 with the amount of isoAsp at position Aβ1 more difficult to estimate due to the variable degree of N-terminal degradation. A third Aβ isoAsp site at position 23 has been reported to accelerate the *in vitro* aggregation kinetics of synthetic Aβ1-42 **[38-40]**. Interestingly, the Aβ mutation at position 23 Asp→Asn (Iowa) produces heavy vascular amyloidosis associated with dementia and intracerebral hemorrhages. In this form of familial AD, an isoAsp at position 23 is produced by deamidation of the mutant Asn residue to Asp followed by isomerization, again via a succinimide intermediate **[41-43]**. The structural resemblance of isoAsp and Asn residues described above may provide some insight into the pathology associated with the Aβ23 Iowa mutation. Another Aβ mutation reported at position Aβ7 Asp→Asn (Tottori) alters the conformational dynamics of Aβ, accelerates the rate of oligomerization and affects metal interactions **[44-47]**.

While immunohistochemical studies suggest that the isoAsp at position 23 is mainly associated with the vascular amyloid deposits, the isoAsp at position 7 appears to be abundant in both parenchymal plaque and vascular related amyloid **[42,43,48]**. These studies also confirmed that in AD subjects the Asp residues at position 1, 7 and 23 are partially isomerized. The preferential localization of isoAsp at position 23 in vascular deposits of Aβ suggests the isomerization event occurs prior to its vascular deposition, soon after Aβ formation. Alternatively, the physicochemical conditions in the vascular compartment may favor the isoAsp23 modification. Conversion of Asp23 to isoAsp alter the kinetics of polymerization and may promote propagation of amyloid in the AD brain **[41]**. Recent cryo-electron microscopy (cryo-EM) observations permitted the 3D-structural reconstruction of the Aβ42 amyloid filaments **[49]**. The model predicts that the negatively charged C_β carboxyl group of Asp23 hinders a more advantageous packing in the stacking of Aβ42 dimer interfaces. Decreasing electrostatic repulsion between adjacent Asp residues will result in a more stable filamentous structure. The formation of IsoAsp may mimic the Asn23 Iowa mutation by displacing the C_β side chain carboxylate to the $23C_\alpha$.

We propose that Aβ isoAsp at positions 7 and 23 in the AD brain may induce conformational changes analogous to the Tottori and Iowa Aβ mutations which are localized at the same positions of the Aβ peptide and associated with early onset AD. These alterations cause drastic changes in secondary and tertiary structure of the Aβ that may facilitate toxicity, seeding and propagation, perhaps by serving as templates converting unmodified Aβ species into self-transmissible amyloid species. It has been reported that reversion of isoAsp into Asp occurs in Aβ in the presence of PIMT and the methyl donor S-adenosyl methionine, resulting in the partial blockade of Aβ fibrillogenesis **[50]**. IsoAsp PTM are undetectable by routine mass spectrometry, since the Aβ peptides with IsoAsp alterations have an atomic mass identical to native Aβ-containing Asp residues. However, estimation of isoAsp can be performed by the enzymatic methods published by Dai et al. **[57],** Tomidokoro et al. **[43]** or by electron capture dissociation combined with Fourier transform mass spectrometry **[51]**. In addition, using a combination of HPLC and mass spectrometry, it is possible to simultaneously determine both racemization and isomerization in Aβ **[52]**. The conformational changes induced by Aβ PTM, alone or in combination, could also mimic the stereochemical disturbances elicited by known deleterious familial AD amino acid substitutions such as Ala21→Gly (Flemish), Glu22→Gln (Dutch), Glu22→Gly (Artic), Glu22→Lys (Italian), in addition to the Asp23→Asn (Iowa) and Asp7→Asn (Tottori), mutations described above. The transition of the peptide bonds from C_{α}-C_{α} to C_{β}-C_{α} carbons, drastically reorients the carboxylate and amino groups which alters the conformation of Aβ peptides and their isoelectric points. This facilitates the generation of β-pleated sheets **[53-56]** thereby rendering these molecules more stable and resistant to enzymatic degradation **[57,58]**. Interestingly, while the isoAsp at position Aβ1 blocks BACE-1 β-secretase hydrolysis, cathepsin B activity efficiently hydrolyzes peptides with isoAsp at this position **[57]**. Additionally, it has been reported that a membrane bound β-secretase can cleave in the presence of a D-Asp residue **[59]**. IsoAsp modifications disrupt the ordered assembly of the α-helix by affecting the stability of the intra- and inter-molecular interactions such as hydrogen bonding, salt bridges and hydrophobic interactions, in turn accelerating rates of Aβ oligomerization and fibril formation **[41,43,46]**. These observations strengthen the contention that Aβ isoAsp isomerization is a potential triggering mechanism for AD amyloidosis and Aβ neurotoxicity.

Pyroglutamate modification

Amyloid-β species containing pyroglutamate at position 3 (Aβ3pE) have been identified in parenchymal plaques, vascular deposits **[60,61]**, presynaptic sites **[62]** and lysosomes **[63]**. About 50% of the Aβ peptides present in purified amyloid plaque cores and about 11% of the total Aβ mass in isolated vascular amyloid deposits have N-terminal Aβ3pE **[64]**. The formation of Aβ3pE requires the removal of the first two N-terminal Aβ amino acid residues followed by the action of the enzyme glutaminyl cyclase **[65]**. Numerous investigations have revealed the presence of this peptide in Aβ deposits, its intrinsic physical properties such as resistance to degradation, fast aggregation rate, increased neurotoxicity, association with behavioral deficits, capacity to form hybrids with other Aβ species as well as its potential role in AD pathogenesis **[65-86]**. Antibodies against the Aβ3pE modified peptide tested in transgenic (Tg) mouse

models decreased Aβ deposits, inhibited Aβ aggregation and reduced behavioral dysfunction **[87-89]**. It has been proposed that the Aβ3pE peptide could be a potential seeding template of highly neurotoxic Aβ **[69,81,90]**. Of the many Aβ PTM, only one, Aβ3pE, has been targeted by immunotherapy and is currently in phase-1 clinical testing by Eli Lilly. Unfortunately, this antibody apparently evoked an undesirable immunogenic response in immunized individuals (see: Fagan T. Alzforum News, AAIC-Toronto, 2016, August 24, 2016).

Phosphorylation

Phosphorylation of Aβ at Ser8 by protein kinase-A **[91,92]** enhances aggregation and toxicity. Phosphorylation of Aβ at Ser26 by human cyclin-dependent kinase-1 has also been reported to increase Aβ toxicity **[93,94]**. It is possible that Ser phosphorylation has been overlooked because the often employed solubilization process utilizes formic acid which readily hydrolyzes esterified phosphate groups. In addition, several studies have suggested that in the AD brain Aβ L-Ser26 can be converted to D-Ser. This racemization apparently produces toxic Aβ fragments that may play a role in neurodegeneration **[95-97]**.

Oxidation

Oxidation of Aβ at Met35 to sulfoxide (S=O) and sulfone (O=S=O) forms has been the object of intense examination. In AD and mild cognitive impairment, oxidative stress mediated by free radicals instigate protein oxidation, lipid peroxidation and reactive oxygen species (ROS) production conducive to synaptic damage with neuronal and glial demise **[98]**. Met35 appears to regulate copper-catalyzed oxidation and aid in the generation of noxious hydrogen peroxide **[99]**. Electron spin resonance studies have confirmed that Met35 intervenes in free radical production. Substitution of Met35 with Val or Leu residues eliminates free radical production, oxidative stress and hippocampal toxicity of Aβ **[98,100,101]**. Furthermore, induction of Met-sulfoxide reductase in Tg mouse models protected neurons from Aβ toxicity **[102]**. Circular dichroism, thioflavine-T and atomic force microscopy methods indicated that AβMet35-sulfoxide impedes fibril formation **[103-105]**. Apparently, the presence of oxidized Met35 favors monomers and dimers over larger oligomers and enhances neurotoxicity **[106]**. Molecular dynamics simulations of Aβ suggest that Met35 oxidation decreases the β-strand content of the C-terminal hydrophobic domain of Aβ, specifically at the Aβ33-35 structural domain and that this configuration hinders Aβ polymerization **[107]**.

Nitrosylation

Nitration at Tyr10 accelerates Aβ aggregation and has been detected in the amyloid plaques of both APP/PS1 mice and AD brains **[108]**. In a more recent study Aβtyr10 was found to significantly decrease Aβ aggregation and cytotoxicity [109].

The intriguing role of dimeric Aβ in AD pathology.

In the 1990s the hypothetical cause of AD pathogenesis shifted from the insoluble fibrillar amyloid plaques to soluble oligomeric forms of Aβ. Substantial work has been dedicated to understanding the physicochemical properties of Aβ aggregates ranging from dimers to large conglomerates **[110-114]**. In 1996, our group isolated detergent-

free, water-soluble Aβ (n-40 and n-42) from normal and AD brains **[111]** in which the most prevalent and stable fraction was dimeric Aβ **[112]**. Amyloid-β dimers derived from AD amyloid plaques and vascular deposits were tested for toxicity in cultures of rat hippocampal neurons and glial cells **[112]**. Intriguingly, Aβ dimers elicited neuronal killing only in the presence of microglia. Amyloid-β dimers with PTM, including isoAsp1 and isoAsp7, cyclization of Glu3 to pyroglutamyl and oxidation of Met35, exhibit increased insolubility and stability. Amyloid-β1-42, with IsoAsp at positions 1 and 7, demonstrated the fastest rate of oligomerization, followed by Aβ3pE-42 and Aβ1-42. Amyloid-β1-40 showed a slower dimerization rate while Aβ1-28 did not dimerize **[58]**. Furthermore, tryptic digestion resistance progressively increases from Aβ1-40 monomer, Aβ1-42 monomer, Aβ3pE-42 monomer, Aβ1-42 (1,7 isoAsp) monomer, Aβ1-42 (1,7 isoAsp) dimer and Aβ17-42. Amyloid-β1-42 with oxidized Met35 to either Met sulfone or sulfoxide, was ~50% more resistant to digestion than non-oxidized Aβ1-42 **[58]**. These experiments suggest that the length of the Aβ peptides and PTM induce structural changes which impart unique physicochemical properties and functional effects.

Several dimeric and oligomeric Aβ models have been investigated in recent years (reviewed in reference **[1]**). Dimeric Aβ based on FASTA and BLAST SwissProt data using the PredictProtein and TOPITS algorithms yielded a Greek-key Aβ motif conformation in which four antiparallel β-strands generate a compact Aβ dimer with a hydrophobic core to shelter non-polar residues from the surrounding water **[115]**. In this model, the hydrophobic C-terminal domains of the Aβ dimer are thermodynamically shielded since they are partially buried along the dimer crevices, but can be extended to form the core of antiparallel β-sheets (see below). This model was further refined by molecular dynamics simulations **[115]**. Atomic force microscopy of purified dimers from amyloid plaques revealed the Aβ dimer as a compact globular hydrated structure ~35-38 Angstroms in diameter **[112,115]**. A series of studies suggests the importance of the stable soluble Aβ oligomers in AD cognitive dysfunction **[115-118]**, conformational-dependent mechanisms of neurotoxicity **[119]**, ability to induce tau hyperphosphorylation and neuronal degeneration **[120]** as well as stability in SDS solutions **[33]** with the latter property implicated in the generation of concentration-dependent dimers **[121]**. However, dimers have been purified in our laboratory in the absence of detergents **[111]**. Amyloid-β dimers isolated from the human brain impair synaptic plasticity and are detrimental to memory by inhibiting long-term potentiation, enhancing long-term depression and decreasing dendritic spine density in animal models **[122]**. Moreover, the degree of neurotoxicity is apparently dependent on the amount of Aβ dimers/trimers **[123]**. Recent experiments suggest that the binding of interstitial fluid Aβ oligomers to GM1 gangliosides produces destabilizing structural changes in membranes **[124]**. Synthetic dimeric Aβ inhibits mitochondrial cytochrome C-oxidase in the presence of copper **[125]**. Single-molecule atomic force microscopy experiments indicate that aggregation of Aβ is modulated by local environmental conditions and that Aβ42 dimerization is an extremely rapid process. In addition, the drastic structural differences between Aβ40 and Aβ42 may play a key role in dimerization propensity **[126,127]**. Amyloid-β dimers have also been proposed as the molecular unit in the polymerization of amyloid fibrils. In this model based on cryo-EM, two opposing monomeric Aβ molecules comprising Aβ residues 25-41 generate a face-

to-face antiparallel β-sheet by adopting an S-shape zipper-like hydrophobic core 'C-domain' while leaving the N-terminal regions, mostly composed of polar amino acids (residues 1-24), to make two opposing 'P-domains'. The subsequent stacking of these dimeric structures creates coiled two-stranded amyloid filaments **[49]**. It has been estimated that Aβ dimers are a million-fold more thermodynamically stable than disordered unstructured Aβ monomers **[126]**.

The role of soluble oligomeric Aβ peptides

In recent years oligomers have been assumed to be the ultimate cause for synaptic dysfunction, neuroinflammation, neurovascular compromise and neuronal/glial degeneration, making them the target of intense research and immunotherapy interventions **[19,21,128-133]**. However, the notion of soluble oligomeric Aβ toxicity still deserves further scrutiny and comprehensive validation. One major problem is that the enormous diversity of the Aβ peptides influenced by PTM and peptide length also affects the size, biochemistry and biophysical properties of oligomers. Although Aβ dimers appear to be stable, larger Aβ oligomers have been isolated from mice and human brains using a variety of purification techniques. Oligomers might assume a very large number of conformational structures with a correspondingly huge diversity of epitopes. This complexity may explain why immunotherapies with antibodies assumed to be reacting with oligomers in the human brain have yielded poor results in clinical trials (reviewed in ref: **[134]**. There is no doubt that variable amounts of soluble monomeric and oligomeric Aβ exist in the human brain because metastable monomeric Aβ is continuously generated from APP by the action of secretases. There is also proof that, at least under controlled experimental conditions, oligomers are neurotoxic in cell culture and experimental animals **[135-140]**. However, the definition of Aβ oligomers is vague since different laboratories in academia and commercial settings produce their own unique varieties based on synthetic peptides and *in vitro* aggregation conditions. In most instances these oligomers, primarily built on unmodified full-length synthetic Aβ40 or Aβ42 amino acid sequences, have been assumed to be a faithful representation of what is present in the far more complex AD brain environment. In addition, Aβ oligomers have been extracted from animal or human brains using techniques that employ a diversity of mechanical homogenizing stresses. These extracted species may include artifacts from dispersed fibrillar Aβ which may not be present in the AD brain. Oligomer with variable states of aggregation may incorrectly indicate a higher concentration of oligomers than actually present.

The complicated catalog of APP/Aβ-related peptides and AD amyloidosis

The profusion of amyloid plaques and their multiple morphological presentations suggests an underlying complexity in chemical compositions. A substantial mass of the amyloid plaque core is composed of a **complex** mixture of glycoproteins, glycolipids, lipids and proteins other than APP/Aβ **[141,142]**. Among the best characterized molecules are a variety of glycosaminoglycans, gangliosides, cholesterol, fatty acids, triglycerides, α1-antichymotrypsin and apolipoprotein E **[143-151]** and a large number of proteins identified by mass spectrometry **[142,152]**. Approximately 35% of the mass of AD amyloid cores is composed of non-Aβ molecules [31] enmeshed within an array of

10 nm fibrillar Aβ peptides. The biological function of the non-Aβ molecules in the context of plaque pathology and dementia has never been investigated in detail. Based on the conventional notion that in AD amyloid plaques are mainly composed of unmodified Aβ1-40 and Aβ1-42 peptides, several therapeutic antibodies have been synthesized against short consecutive amino acid sequences of the intact N-terminal, C-terminal and middle domains of these peptides. Biochemical analyses of AD purified amyloid plaque cores have shown that the N-termini of Aβ are highly variable, probably resulting from aminopeptidase activity that is associated with degradation pathways of Aβ. In addition, BACE1, that normally cleaves APP to generate the amino terminus of Aβ1-40/42, can also cleave APP at residue Aβ11 to generate Aβ11-40/42 **[153]**. The proteolytic activity of the α-secretase on APP produces the "non-amyloidogenic" Aβ17-40/42, recognized as P3, which is abundant in diffuse amyloid plaques in cortical and cerebellar deposits **[154-156]**. These plaques have been deemed "non-fibrillar" but are known from thioflavine-S staining and EM studies to contain a low density of amyloid fibrils **[157]**. Due to its overall hydrophobic composition and insolubility P3 is very difficult to test in cell and animal models leaving the function of this peptide still unknown. However, because it is associated with diffuse plaques and may not elicit adjacent inflammatory reactions, P3 has been assumed to be an innocuous molecule. The potential ability of P3 to disrupt membrane lipids and form ionic channels implies this peptide may induce pathological changes in membrane permeability **[158-160]**.

The Aβ C-termini are also variable **[161]**. It has been proposed that the ɣ-secretase primarily cleaves APP at residues Aβ48 and Aβ49, known as ε-sites, producing Aβ1-48 and Aβ1-49, and corresponding intracellular domains (AICD) 49-99 and 50-99 **[162,163]**. In addition, the ɣ-secretase can hydrolyze APP at residues Aβ46-47, the ζ-site **[164]**, thus generating longer Aβ peptides **[165-167]**. The sequential hydrolysis of APP by ɣ-secretase in AD apparently generates a step-wise series of Aβ peptides terminating in residues 49, 48, 46, 45, 43, 42, 40, 39, 38 and 37 **[162,163].** These Aβ forms have not been quantified in the AD brain. It is likely that the ratios of these Aβ peptides will vary from individual to individual. Interestingly, in the *PSEN1* EOAD mutation E280A (paisa) the Aβ C-termini are also heterogeneous with peptides ending at every position from residue 42 to residue 55 **[168]**.

The traditional view that concerted processing of APP by the α, β and ɣ secretases produces Aβ amyloidogenic and non-amyloidogenic peptides is complicated by the recognition of alternative APP cleavage sites **[169]**. Some elongated Aβ-related peptides have been isolated and rigorously characterized by amino acid sequencing. Amyloid precursor protein hydrolysis at the δ- position Thr584 (APP_{695}) yields a product with an additional 12 amino acid residues extending from the N-terminus of the Aβ peptide **[170]**. More recently, two additional APP/Aβ peptides produced by an asparagine endopeptidase have been identified. Cleavage of APP_{695} at Asn373 creates an APP N-terminal neurotoxic peptide, and at Asn585 yields an APP C-terminal peptide, composed of residues 586-695 that serves as a preferred substrate for BACE1 **[171]**. It was further suggested that this latter peptide increases amyloid production, highlighting the potential importance of the δ-site in AD pathogenesis **[171]**. Another APP hydrolysis site, defined as the η-site, was discovered between residues 504-505 (APP_{695}). The η-peptide is further processed by the β- and α-secretases to create the

Aη-β and Aη-α APP fragments. The latter peptide inhibited neuronal activity in the hippocampus by lowering long-term potentiation **[172]**. It has been suggested that cathepsin-L degrades the η-C-terminal fragment of APP **[173]**. In addition to these APP-derived peptides, the APP C-terminal fragment containing the last 100 amino acids of APP (emulating β-secretase hydrolysis and absence of ɣ-secretase cleavage) induces neurodegeneration in transgenic mice **[174,175]**. Moreover, the AICD fragment can be further hydrolyzed to yield the Jcasp and the C31 peptides that have been found to induce apoptosis and have neurotoxic activity **[176-179]**. Lastly, APP-derived peptide carrying the N-terminal sequence of amino acid residues 18-286 was found to produce axonal pruning and neuronal death by interacting with the death receptor-6 (DR6) via the activation of caspases **[180]**.

The evolutionary conservation of the APP and the redundancy generated by the amyloid precursor like-proteins (APLP1 and APLP2A) molecules is a testimony to its importance in modulating the function and fate of cells. The increased expression of APP is likely to generate an overproduction of specific peptides that may influence AD pathogenesis and development **[181]**.

Implications of the AN-1792 active vaccination clinical trial

Neuropathological and biochemical examination of the brains of individuals actively vaccinated with aggregated synthetic Aβ1-42 + adjuvant (AN-1792) revealed neuritic and cored plaques were apparently disrupted while diffuse plaques and cerebrovascular amyloid were unaffected **[182-186]**. The cerebral cortex of vaccinated individuals showed a distinctive patchy distribution of neuritic and cored plaques with intercalation of adjacent plaque-poor and plaque-rich areas. In some individuals, the amyloid plaques left remnants suggestive of 'collapsed plaques' or 'moth-eaten plaques' that were reminiscent of the putative original plaque outline **[182-186]**. In some other instances, remnant structures exhibited a minuscule central deposit of amyloid surrounded by a clear area devoid of amyloid and a thin peripheral 'halo' of amyloid positive material **[186]**. ELISA analyses revealed the levels of water-soluble Aβ40 and Aβ42 were dramatically increased compared to a non-vaccinated AD population. In addition, vaccinated subjects had increased amounts of formic acid/guanidine hydrochloride-extractable Aβ40 coupled with a decrease in Aβ42 levels **[187]**.

The above data suggest that, in some vaccinated individuals with high serum antibody titers, the anti-Aβ antibodies effectively crossed the blood-brain barrier (BBB) and reached their targets. These antibodies were capable of removing amyloid from plaque neuritic haloes and cores, probably from those mainly containing Aβ42. The interrupted pattern of plaque loss, however, indicates either variability in vascular antibody permeability or of their action on subtypes of amyloid deposits. Additionally, the patchy plaque elimination could be a consequence of treatment cessation since the trial was discontinued after some patients developed aseptic meningoencephalitis. Interestingly, Holmes et al. **[188]** reported that some cases exhibited an almost complete absence of histologically visible amyloid deposits. However, it is likely that some subjects never harbored amyloid deposits in the first place. For instance, case #14, described in reference **[187]**, reported as having a complete absence of plaques had the lowest levels of Aβ formic acid extracted Aβ40 and Aβ42 and no soluble amyloid by

immunoassays. However, this subject was Braak stage VI and likely an instance of a primary tauopathy such as progressive supranuclear palsy or corticobasal degeneration.

AN-1792 active vaccination was apparently far more effective at plaque disruption than passive immunizations with monoclonal antibodies. In the former case, multiple polyclonal antibodies recognized a large number of epitopes generated by different Aβ aggregated conformations. However, in most cases, the clearance of Aβ deposits was incomplete since diffuse plaques rich in Aβ17-42 (P3) and vascular-associated amyloid in cerebral cortex and leptomeningeal vessels, composed primarily of Aβ40, were unaffected. Despite the apparent effectiveness of AN-1792 in disrupting at least some amyloid plaques, this therapy notably failed to halt cognitive impairment progression **[188]**.

Peripheral Aβ

Amyloid precursor protein is expressed in most human cells suggesting peptides derived from this molecule, including Aβ, exist in most tissues and compartments of the body. In addition to the uncertainty over the temporal pace of Aβ deposition and the sequential location of brain affected sites, the role of Aβ in circulating plasma and CSF in the development of AD remains enigmatic. Circulating Aβ is predominately bound to albumin and other plasma molecules **[189-191]**. Amyloid-β has been detected in peripheral tissues **[192]**. For example, in skeletal muscle the levels of Aβ42 and total Aβ are significantly elevated in AD when compared to non-demented controls. Like the brain, skeletal muscle, which represents about one-third of the body mass, also generates a diverse array of Aβ peptides **[193]**. Furthermore, the aortas of elderly individuals with severe atherosclerotic deposits contain twice the amount of total Aβ40 and Aβ42 than subjects with minimal atherosclerotic vascular disease **[194]**. Another important source of peripheral Aβ are the platelets. Quiescent platelets contain more Aβ40 than activated de-granulated ones **[192]**. The administration of anti-Aβ antibody infusions are likely to have some effect on the levels of circulating Aβ generated in peripheral tissues. Hence, any therapeutic interventions against AD amyloidosis relying only on the levels of circulating Aβ levels to measure their efficacy may lead to erroneous interpretations. Whether or not circulating Aβ contributes to the brain pool of these molecules remains to be answered with certainty. The physiologic and health implications of perturbing peripheral Aβ pools on a chronic basis are unknown.

Future biomarker discovery and immunotherapy tactics

While many studies have confirmed the role of Aβ in AD pathology, there is considerable confusion as to which of its myriad forms will provide effective diagnostic markers and therapeutic targets. Numerous lines of evidence have implicated various Aβ species including soluble, oligomeric, globular or annular aggregates **[195-202]** as critical players in synaptic demise and early memory loss of AD. Likewise, there is no consensus regarding the form(s) of covalently modified Aβ most intimately involved in neurological decline. There is also considerable uncertainty over where Aβ accumulation first occurs in the brain and whether the deposited molecules are generated within the brain exclusively or augmented by peripheral pools. Under normal circumstances Aβ is proteolytically degraded in brain or cleared by the liver and kidneys

[203-205], but very little is known about the catabolism of the PTM Aβ peptides. Adding to these complexities, a variety of homogeneous or heterogeneous aggregated Aβ species could be stochastically generated in brain tissue. In some regions of the AD brain up to 12 copies of the APP gene have been found in some neurons. Expression of all or some of these APP genes may **participate** the pathogenesis of AD **[206,207].** Different Aβ peptide species may play distinct **roles that are dictated by their specific molecular conformations.**

Identification of Aβ related antibodies that selectively recognize conformational epitopes in different AD patients is an ideal approach for the development of biomarkers and therapeutic agents. Antibodies against Aβ oligomers have been utilized to confirm the existence and role of oligomeric Aβ species **[117,196,197,208-210]**. The most useful Aβ antibodies for biomarker discovery might be those targeting **specific epitopes on molecules known to be widely distributed in AD subjects.** Novel methods have achieved this goal by combining the imaging capabilities of atomic force microscopy with phage display antibody technology which enables the identification of specific protein variants and isolation of reagents that selectively bind the target protein **[211]**. These technologies permit the generation of antibody based (nanobody) reagents that preferentially differentiate toxic-disease associated variants of key neuronal proteins including Aβ, tau, TDP43 and α-synuclein **[211-220]**. In the case of Aβ, nanobodies revealed three conformationally distinct oligomeric variants that differentiate postmortem AD brain specimens from healthy or Parkinson's disease cases **[219,221-223].** These observations indicate that detection of disease related protein variants may be a powerful blood or CSF based biomarker tool for AD and related neurodegenerative diseases. Since Aβ is such a complex protein and AD is a heterogeneous disease, detection of specific Aβ variants and other related deviant proteins have great promise as individualized biomarkers for AD and great potential for precision-personalized medicine.

Conclusions

At the center of the AD-amyloid conundrum is the unresolved observation that in the absence of genetic mutations Aβ peptides spontaneously aggregate into amyloid plaques and the walls of the cerebral vasculature. We contend this apparently spontaneous change is enhanced by alterations gene expression and PTM of the Aβ peptide structures, increasing their stability and propensity to polymerize.

It is unclear whether the widely accepted assumption that unmodified, full length Aβ40/Aβ42/Aβ43 and their soluble/oligomeric/fibrillary forms are the main culprits responsible for the pathology and clinical manifestations of late-onset AD. Experimental investigations reveal the Aβ molecules harbored by AD subjects are structurally diverse with different conformations and biological properties. **However, to date most passive Aβ immunotherapies, with the exception of aducanumab, have targeted relatively short linear Aβ1-42 amino acid sequences rather than specifically folded tertiary structures.**

Mounting evidence suggests that pathologic prions derived from normal proteins underlie several neurologic diseases including AD. Prion strains exhibit unique biochemical properties imparted by specific toxic molecular conformations. These strain-specific pathologic conformations are faithfully replicated (Watts JC, Condello C, Stohr J, Oehler A, Lee J, DeArmond SJ, Lannfelt L, Ingelsson M, Giles K, Prusiner SB. Serial propagation of distinct strains of Aβ prions from Alzheimer's disease patients (2014), 111, 10323-10328). **Conformational alterations induced by PTM of Aβ to yield unique amyloid strains may partially account for the clinical and pathological heterogeneity of LOAD** (Watts JC, Condello C, Stohr J, Oehler A, Lee J, DeArmond SJ, Lannfelt L, Ingelsson M, Giles K, Prusiner SB. Serial propagation of distinct strains of Aβ prions from Alzheimer's disease patients (2014), 111, 10323-10328). **Analogous to situations in which transmissible prions cross species barriers, the Aβ molecules of AD subjects would be induced to adopt and faithfully propagate the specific toxic conformation of spontaneously emerging pathologic seeds. Self-transmissible Aβ strains capable of inducing distinct pathologic manifestations have been isolated from AD subjects** (Watts JC, Condello C, Stohr J, Oehler A, Lee J, DeArmond SJ, Lannfelt L, Ingelsson M, Giles K, Prusiner SB. Serial propagation of distinct strains of Aβ prions from Alzheimer's disease patients (2014), 111, 10323-10328).

To date, Aβ physical diversity and functional significance of 3D conformations to dementia and toxicity have been disregarded. In addition to these differing biophysical features among Aβ species, quantitative differences in the proclivity to accumulate may also contribute to their pathological oligomerization and deposition in the aging brain. It can be assumed that some of these Aβ-related molecules have positive adaptive functions while others may be detrimental to brain homeostasis. Several lines of circumstantial and experimental evidence have suggested that under damaging conditions such as brain trauma, microbial invasion, a leaky blood-brain barrier and hypertensive crisis, sustained overproduction of some Aβ peptides may have a rescue function. This assumption is supported by the molecular conservation of the Aβ amino acid sequence along mammalian evolution that suggests important adaptive values for these peptides. It is still unclear which Aβ alternatives, including PTM peptides, are involved in the onset and progression of AD and thus might represent the best therapeutic targets, or, alternatively, which may have a salvage function.

We propose that amplified production of some Aβ species, probably complicated by reduced degradation occurring during aging, creates a diverse spectrum of molecules which ultimately disrupt brain homeostasis and contribute to AD neurodegeneration. We postulate that the generation of some specific 3D-peptide conformations of Aβ impart a unique array of biophysical properties with deleterious as well as protective effects. **Proteolytic processing of the highly evolutionarily-conserved multifunctional APP molecule is capable of creating over a dozen of proteolytically-derived peptides which are involved in a large number of brain functions, some of them with deleterious properties.** The APP dynamics must be finely tuned through transcription and translation and closely regulated in terms of proteolytic processing and degradation. In addition to Aβ, the excessive production of multiple neurotoxic peptides derived from the proteolysis of APP may play important roles in the development of late-onset AD.

Some of these APP peptides may be involved in the initial stages of AD and could have profound effects in subsequent neurodegeneration.

One factor confounding interpretation of previous clinical trials is the observation that a large fraction of elderly dementia cases, even those clinically thought to have AD, are not associated with conventionally defined AD neuropathology based on threshold densities and distributions of plaques and tangles **[224]**. The A/T/N classification scheme of Jack *et al*. **[225]** proposes to integrate additional markers of neurodegeneration into the nosological partition of AD and other dementias, helping to define clinical subgroups. Coupled with imaging methods capable of revealing amyloid and tangle deposits in living subjects and correlated with clinical signs and symptoms, this more nuanced view of dementia may aid in the design and interpretation of future clinical trials.

Advances in imaging techniques, genetics and neurochemistry will further enable investigators to classify demented subjects on the basis of amyloid or tau deposition patterns with unprecedented precision. Sophisticated, minimally-invasive biopsy methods **[226],** coupled with innovative analytical techniques would help clarify the effects of Aβ molecular diversity on pathogenesis and aid in the identification of additional pathologies including tau, α-synuclein and TDP-43. Longitudinal studies combining imaging, molecular fingerprinting and cognitive function exams may reveal if the kinetics assumed for the amyloid cascade hypothesis holds for the majority or only a limited number of AD demented subjects. Clarifying which of the structurally altered Aβ peptides are responsible for neurotoxicity will help in the design of specific therapeutic interventions. Reagents that selectively recognize and target different Aβ conformational variants will be powerful tools to assist in the individual diagnosis and personalized treatment of AD patients. Detailed examinations of the non-demented oldest-old subjects retaining cognitive function while harboring the neuropathologic lesions of AD may help reveal which amyloid species are inimical to neuronal and vascular function and which may be comparatively less toxic or non-toxic.

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Competing Interests

Roher AE, Clarke SG, Kokjohn TA, Maarouf CL, Sierks MR and Serrano G, have no conflicts of interest to declare.

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