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REVIEW ARTICLE

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Refining the amyloid β peptide and oligomer fingerprint ambiguities in Alzheimer's disease: Mass spectrometric molecular characterization in brain, cerebrospinal fluid, blood, and plasma

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Abstract

Since its discovery, amyloid-β (Aβ) has been the principal target of investigation of in Alzheimer's disease (AD). Over the years however, no clear correlation was found between the Aβ plaque burden and location, and AD-associated neurodegeneration and cognitive decline. Instead, diagnostic potential of specific Aβ peptides and/or their ratio, was established. For instance, a selective reduction in the concentration of the aggregation-prone 42 amino acid-long Aβ peptide (Aβ42) in cerebrospinal fluid (CSF) was put forward as reflective of Aβ peptide aggregation in the brain. With time, Aβ oligomers—the proposed toxic Aβ intermediates—have emerged as potential drivers of synaptic dysfunction and neurodegeneration in the disease process. Oligomers are commonly agreed upon to come in different shapes and sizes, and are very poorly

Abbreviations: aa, amino acid; ACE, angiotensin-converting enzymes; AD, Alzheimer's disease; ADAM, a disintegrin and metalloproteinase domain-containing protein; APOE, apolipoprotein E; APP, amyloid precursor protein; Aβ, amyloid-β; BACE, beta-site amyloid precursor protein cleaving enzyme; CAA, cerebral amyloid angiopathy; CR, Congo Red; cryo-EM, cryogenic electron microscopy; CSF, cerebrospinal fluid; CTF, C-terminal fragment; CU-AP, cognitively unaffected-amyloid positive; DS, Down syndrome; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FA, formic acid; fAD, familial AD; GBSC, Global Biomarker Standardization Consortium; IDE, insulin-degrading enzyme; IFCC WG-CSF, International Federation of Clinical Chemistry and Laboratory Medicine Working Group for CSF proteins; IMS, imaging mass spectrometry; IP, immunoprecipitation; JCTLM, Joint Committee for Traceability in Laboratory Medicine; LC, liquid chromatography; LCO, luminescent conjugated oligothiophene; MALDI, matrix-assisted laser desorption/ionization; MMP, matrix metalloproteinase; MS, mass spectrometry; NEP, neprilysin; NTE, N-terminally extended; PAGE, native polyacrylamide gel electrophoresis; PDMS, plasma desorption mass spectrometry; PET, positron emission tomography; pGlu, pyroglutamate q-FTAA, quadro-formylthiophene acetic acid; PICUP, photo-induced cross-linking of unmodified proteins; PSEN, presenilin; PTM, post-translational modification; sAD, sporadic AD; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; SELDI, surface-enhanced laser desorption/ionization; SILK, stable isotope labeling kinetics; Simoa, single-molecule array; SPE, solid phase extraction; ssNMR, solid-state nuclear magnetic resonance; TBS, Tris-buffered saline; WB, western blot.

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characterized when it comes to their composition and their "toxic" properties. The concept of structural polymorphism—a diversity in conformational organization of amyloid aggregates—that depends on the Aβ peptide backbone, makes the characterization of Aβ aggregates and their role in AD progression challenging. In this review, we revisit the history of Aβ discovery and initial characterization and highlight the crucial role mass spectrometry (MS) has played in this process. We critically review the common knowledge gaps in the molecular identity of the Aβ peptide, and how MS is aiding the characterization of higher order Aβ assemblies. Finally, we go on to present recent advances in MS approaches for characterization of Aβ as single peptides and oligomers, and convey our optimism, as to how MS holds a promise for paving the way for progress toward a more comprehensive understanding of Aβ in AD research.

1 | INTRODUCTION

Amyloid β (Aβ) peptides and their aggregation into insoluble amyloid fibrils in the form of extracellular Aβ plaques in the brain is the central hallmark of Alzheimer's disease (AD) (Jack et al., 2018). Since the discovery of Aβ peptides in cerebrovascular amyloid deposits in the mid-1980s (Glenner & Wong, 1984a, 1984b; Masters et al., 1985), and the full Aβ amino acid (aa) sequence from amyloid plaque cores in 1992 (Mori et al., 1992) extensive research has been done aiming to characterize the diversity of both non-aggregated Aβ peptides (monomeric Aβ) and the diversity of primary, secondary, and tertiary structures of Aβ in AD plaques in order to understand the onset and progression of the disease.

The focus on Aβ aggregation and its interplay with downstream events as the driving force, or the trigger in AD, was put forward in 1991 with the amyloid cascade hypothesis (Beyreuther & Masters, 1991; Hardy & Allsop, 1991; Hardy & Higgins, 1992; Selkoe, 1991a, 1991b). The fact that Down syndrome [DS; trisomy 21; the chromosome where the amyloid precursor protein (APP) gene is located] is characterized by progressive accumulation of Aβ pathology with age, as well as the discoveries of multiple mutations that alter APP processing and Aβ peptide homeostasis, are some of the aspects used for the support of the amyloidocentric view on AD pathogenesis. Selective reduction in the concentration of the aggregation-prone 42 amino acid long Aβ (Aβ42) in CSF, as reflective of Aβ peptide aggregation in the brain (Strozyk et al., 2003), is widely used as a reliable biomarker for Aβ plaque pathology. Recent data suggest that this can also be monitored in blood, particularly in the form of a reduced ratio of the concentrations of 42 to 40 aa long Aβ (Aβ42/Aβ40) (Cohen et al., 2019).

Still, over the course of the years, no correlation was found between the Aβ plaque pathology burden as such, and cognitive symptoms in AD (Brettschneider et al., 2015; Jucker & Walker, 2013; Thal et al., 2002). However, it was shown that Aβ plaques correlate with neuroinflammation and neuritic dystrophy (Dickson, 1997; Eikelenboom & Stam, 1982; Griffin et al., 1989; McGeer & McGeer, 2013; Tsai et al., 2004). It also became apparent that the Aβ pathology was present in non-demented [referred to as cognitively unaffected-amyloid positive (CU-AP)] subjects (Delaere et al., 1990; Dickson et al., 1992). Importantly, the majority of clinical trials where anti-Aβ therapies targeting monomeric Aβ peptides have been used, or those trying to lower the Aβ plaque burden, have failed (Karran et al., 2011; Mehta et al., 2017). As a result, the relevance of the Aβ peptides and particularly the end form of their aggregation, the Aβ plaques, were questioned.

Given this unclear role of Aβ peptides in AD pathogenicity, a need to explicate the neurotoxicity of Aβ became critical. Soluble Aβ oligomers, a set of intermediate aggregation species; rather than Aβ monomers or full-grown Aβ fibrils present in plaques, have been suggested to be the underlying cause of synaptic dysfunction and cell death in AD (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999b). However, the existence of a specific toxic Aβ oligomer has been hard to verify and, rather than single species, a large diversity in shapes and sizes of both synthetic and native Aβ assemblies, with various neurotoxicity, has been reported [for detailed reviews see (Benilova et al., 2012; Haass & Selkoe, 2007; Selkoe, 2011; Shankar & Walsh, 2009; Walsh & Selkoe, 2007)]. At the same time as the work on oligomer toxicity has continued, a concept of structural polymorphism, a diversity in conformational organization of amyloid aggregates, has emerged (Fändrich et al., 2018; Jonson et al., 2019; Rasmussen et al., 2017; Tycko, 2015). Such polymorphism, proposed to occur because of distinct Aβ peptide composition, is indeed present both between different mutations in familial AD (fAD), but also between individual plaques and/or between other Aβ assemblies in sporadic AD (sAD) (Michno, Nystrom, et al., 2019; Rasmussen et al., 2017). This poses the question, how does one precisely characterize the species in the soluble Aβ assemblies, and define their mechanistic properties?

The common approach to separate small and large (low and high molecular weight, respectively) oligomers from one-another is to use native polyacrylamide gel electrophoresis (PAGE), but also sodium dodecyl sulfate PAGE (SDS-PAGE) (Burdick et al., 1992; Hilbich et al., 1991; Walsh et al., 1997). To separate so-called protofibrils from even larger aggregates, size-exclusion chromatography (SEC) is

used (Hilbich et al., 1992; Soreghan et al., 1994; Walsh et al., 1997). However, precise characterization and quantification of these species are problematic with these approaches. Likewise, optimistically one might envision the development of specific Aβ oligomer, protofibril, and fibril antibodies (Kayed et al., 2003, 2007, 2010; Kayed & Glabe, 2006; Lesné et al., 2006). Such an approach, while possibly aiding in general screening, cannot delineate highly complex Aβ assemblies. Besides the inherent issues of immunoassays, at least when using only one antibody, one can neither precisely nor directly characterize single amino acid differences, post-translational modifications (PTMs), or molecular interactions underlying secondary and tertiary structures of Aβ aggregates.

With time, it became apparent that mass spectrometry (MS) might be one of the most suitable tools capable of aiding characterization of oligomers, including individual Aβ peptide constituents, their modifications, interactions between multiple monomeric Aβ peptides, and the structural polymorphism at large (Grasso, 2019; Kummer & Heneka, 2014; Michno, Nystrom, et al., 2019).

Although many of the discoveries involving Aβ have been made without the use of MS, there are also several examples where MS has either contributed or been crucial. For example, Mori et al. used MS to demonstrate that not only is Aβ1-40 the main peptide in the brain, but did also show the presence of N-terminally truncated, pyroglutamate containing (pGlu) Aβ peptides (pGlu Aβ3-x) (Mori et al., 1992). Another example is the mapping of glycosylation sites both for Aβ and NTE-Aβ (including the first published Tyr-glycosylation in mammals) was performed with MS (Halim et al., 2011). Furthermore, although there were very strong indications that dimers existed in brain, the final proof that covalently linked Aβ dimers exist in brain also required MS, and the particular link would have been impossible to determine with other methods (Brinkmalm et al., 2019).

The Aβ turnover is another example where MS has provided new information (Patterson et al., 2015). Although most of the knowledge on Aβ has been acquired without MS in the particular studies, MS has in many cases solidified these findings (earlier or later) by verifying the presence of Aβ in general or by detecting specific Aβ variants. It has also been utilized to delineate/clarify false findings, such as potential dimers/oligomers in cell media or CSF and proven that these are NTE-Aβ or sAPPα fragments (Grant et al., 2019). While to date, direct measurements of Aβ oligomers with MS are not possible to the same degree as, for instance, by cryogenic electron microscopy (cryo-EM) (*e.g.*, no structural identification can be achieved) and to be performed directly in vivo (*e.g.*, using oligomer-specific antibodies), MS enables measurements of chemical and physical properties of various oligomers (*e.g.*, Asp *vs*. isoAsp, retention time differences, or collision cross section), if changes in monomeric Aβ are sufficiently significant (Bleiholder et al., 2011; Dammers et al., 2017; Gremer et al., 2017; Kayed & Glabe, 2006; Yang et al., 2011).

In this review, we will revise the nature of the Aβ peptide, with its complex diversity and properties and the concept of Aβ assembly formation as a result of Aβ misfolding, focusing on intermediate soluble Aβ species. We will contextualize this aggregation in relation to Aβ peptide backbone differences associated with mutations and the underlying peptide length. We will not review the toxicity of the Aβ oligomers, as detailed reviews of this aspect have recently been published (Benilova et al., 2012). Instead, we will outline the contribution of MS in the progression of Aβ centric research and oligomer characterization. We will revise common misconceptions and knowledge gaps in the molecular identity of the Aβ peptide, and higher order Aβ assemblies. Finally, we will present recent advances in MS approaches for characterization of Aβ as single peptides and oligomers, but also for studies of the interactions, PTMs, and turnover kinetics [*e.g.*, stable isotope labeling kinetics (SILK)].

2 | Aβ GENERATION, HOMEOSTASIS, AND MISBALANCE IN ALZHEIMER'S DISEASE

Aβ peptides are generated through sequential proteolysis of the receptor-like transmembrane protein, the amyloid precursor protein (APP), with the most commonly expressed isoforms APP695, APP751, and APP770 (Kang et al., 1987; Yoshikai et al., 1990), along the amyloidogenic pathway (Figure 1a). First, the β-site APP-cleaving enzyme, beta-site amyloid precursor protein cleaving enzyme 1 (BACE1 *i.e.*, β-secretase), cleaves off the extracellular component of APP, generating the N-terminus of the Aβ peptide (Hampel et al., 2021). From the C-terminal end of the APP protein, the C-terminal fragment is initially subject to ε-cleavage either at threonine (aa 48) or leucine (aa 49). Then the γ-secretase complex mediates cleavage of the remaining intramembrane fragment at the C-terminal aa 38, 40, 42, and 43 (γ-sites) generating fragments that are released extracellularly (De Strooper, 2003). Preferential production of Aβ1-40 from cleavage at leucine (aa 49) or Aβ1-42 from the threonine (aa 48) cleavage with consecutive loss of tripeptides has been proposed (Chen et al., 2014; Funamoto et al., 2004). However, every fourth, fifth, and sixth amino acid cleavages have also been suggested (Matsumura et al., 2014). Importantly, the Aβ1-38 truncation, which is less aggregation-prone and even anit-amyloidogenic, has been shown to be independent of Aβ1-42 (Czirr et al., 2008; Page et al., 2008).

Nevertheless, while it is clear that multiple cleavage sites in APP exist, in particular at the C-terminus, the γ-site cleavage appears to be the primary factor influencing the self-aggregation of the Aβ peptide. Here, the most hydrophobic of these Aβ peptides, the one ending at aa 42 (Aβx-42), is considered the primary aggregation-prone peptide in vivo. Alternative processing, referred to as non-amyloidogenic pathways, gives rise to additional peptides. Initial cleavage by α-secretase followed by γ-secretase cleavage produces the p3 fragment (Aβ17-40/42) and a combination of cleavage by β-secretase and α-secretase produces short N-terminal Aβ peptides (Aβ1-15/16). In addition, combinations of α- or β-secretase with δ- and η-secretase produce different peptides extending N-terminally of the β-site.

Most Aβ studies have concentrated on the neurotoxic role of Aβ peptides (particularly Aβ42) because of their central role in AD. The peptides have, however, been proposed to have multiple physiological functions, playing a role in neurogenesis, calcium homeostasis,

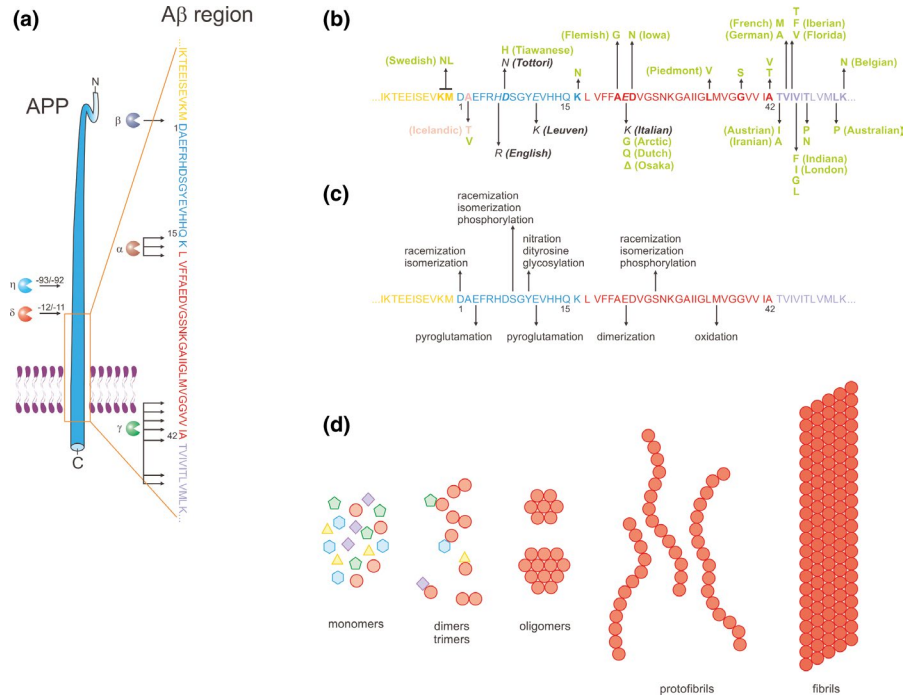

FIGURE 1 Aβ generation, primary sequence, post-translational modifications, and aggregation. (a) A schematic of APP indicating the most common cleavage sites, and an expanded illustration of the Aβ sequence and flanking amino acids. The amino acid sequence of Aβ is shown in blue and red flanked by residues N- and C-terminal (gold and purple, respectively) of APP. (b) A double mutation immediately N-terminal to the Aβ domain of APP, 12 mutations within the Aβ sequence, and at least 15 mutations within 15residues of the A713 C-terminus of the Aβ domain are associated with confirmed cases of fAD or familial cerebral amyloid angiopathy (CAA) and are indicated in bold. For four of the mutations identified within the Aβ domain (shown in *italic*), the pathogenicity has yet to be confirmed. The A673T variant which results in the substitution of alanine by threonine (shown in pink) is the only known amino acid change thought to protect against AD and is associated with minimal amyloid deposition and a reduced incidence of dementia. (c) At least 12 different post-translationally modified forms of Aβ have been identified in biological samples. (d) A schematic of Aβ aggregation. Unstructured Aβ monomers may sample a multitude of transient structures some of which facilitate aggregation into thermodynamically stable fibrillar and/or amorphous aggregates

modulation of synaptic activity, and plasticity (Brothers et al., 2018; Dawkins & Small, 2014; Shoji et al., 1992). Their production is normally balanced by clearance because of enzymatic degradation by multiple proteases (Saido & Leissring, 2012). Their activities depend on multiple factors, such as their subcellular localization or optimal working pH (Caccamo et al., 2005; Saido & Leissring, 2012).

Different proteases are responsible for the degradation of specific Aβ substrates, including non-aggregated and/or aggregated Aβ (Saido & Leissring, 2012). For instance, the insulin-degrading enzyme, angiotensin-converting enzymes (ACE), and neprilysin are responsible for degrading the non-aggregated forms of Aβ (Hu et al., 2001; Saido & Leissring, 2012; Saito et al., 2003). In this context, site-specific serine phosphorylation (aa 8), has been shown to decrease Aβ degradation by the insulin-degrading enzyme and ACE (Kumar et al., 2012; Rezaei-Ghaleh et al., 2016b).

On the other hand, aggregated forms of Aβ are degraded by matrix metalloproteinase-2 and -9, the cysteine protease cathepsin B, and the aspartyl protease cathepsin D (Saido & Leissring, 2012; Saito et al. 2003). The efficiency with which each of these enzymes functions and manages to clear the Aβ peptides (or their aggregates) depends on its respective dynamic equilibrium between various interconnected compartments, and between its passive and active transport (Saido & Leissring, 2012). Combined, all these factors will affect the overall concentration of the Aβ peptide (and possibly the relative concentrations of its proteoforms, Aβx-38, Aβx-40, Aβx-42), as well as Aβ aggregates (*e.g.*, oligomers) at different locations.

Disruption of the Aβ homeostasis was initially proposed to underlie the symptoms of the non-genetic sAD in the amyloid cascade hypothesis (Selkoe, 2001; Tanzi et al., 2004). Such misbalance could either stem from elevated production or from inadequate clearance, resulting in excessive Aβ accumulation and deposition in the forms of Aβ plaques. Indeed, already in the early stages of AD research, it was shown that multiple factors could affect either the protease-dependent production or clearance, including primary peptide sequence, overall or relative concentrations of distinct peptides (Burdick et al., 1992), pH (Fraser et al., 1991), PTMs (Mori et al., 1992; Näslund et al., 1994), as well as interactions with lipid membranes (Arispe et al., 1993; Di Paolo & Kim, 2011) or metal ions (Bush, 2013; Bush & Tanzi, 2008).

3 | FROM fAD TO sAD: Aβ BACKBONE DIVERSITY AND ISOFORMS

While the etiology of idiopathic (commonly known as "sporadic") AD still remains unknown, the pathological features and clinical

symptoms of sAD and fAD are similar. Therefore, despite the initial cause remaining unknown, the fAD mutations might provide the key to understanding the role of Aβ peptides in sAD.

To date, nearly 60 mutations in the APP gene are known (and over 300 in presenilin-1 (*PSEN1*) and over 50 in *PSEN2*). The effects of some of these mutations are not clear and some are not pathogenic. However, when exhibiting a phenotype, these mutations lead to disruption of Aβ homeostasis. This occurs either through an increase in general Aβ production - if the mutations are located N-terminally of Aβ (*e.g.*, the Swedish mutation, KM670/671NL) (Cai et al., 1993; Citron et al., 1992; Kumar-Singh, 2008; Mullan et al., 1992); an increase in the propensity of Aβ peptide to aggregate - if the mutations are within Aβ sequence (*e.g.*, the Arctic mutation, E693G) (Kamino et al., 1992; Kumar-Singh, 2008; Nilsberth et al., 2001); or through an increase in the relative amount of Aβ1-42 production compared with less aggregation-prone Aβ peptides, such as Aβ1-40, when modifying the C-terminal processing (*e.g.*, the London mutation V717I in APP, or PSEN mutations) (De Jonghe et al., 2001; Duff et al., 1996; Eckman et al., 1997; Goate et al., 1991; Herl et al., 2009; Kelleher & Shen, 2017; Kumar-Singh, 2008; Zoltowska et al., 2016) (numbering is according to APP770, Figure 1c). Recently, another protective mutation, APP A673T, known as the Icelandic mutation, was reported (Jonsson et al., 2012). Together, this highlights the significance of single amino acid differences in the Aβ homeostasis, particularly in the context of the major Aβx-40 and Aβx-42 peptides.

Still, while the primary focus of the field has for many years centered around these peptides, a highly diversified Aβ peptide proteoform composition, that is, other C- and N-terminally truncated peptides, have been identified in brain tissue (Brinkmalm et al., 2019; Gkanatsiou et al., 2019; Portelius, Bogdanovic, et al., 2010; Wildburger et al., 2017), CSF (Brinkmalm et al., 2012; Portelius et al., 2006; Rogeberg et al., 2015) and recently also blood/plasma (primarily in sAD patients) (Kaneko et al., 2014). Likewise, Aβ peptides have been shown to exhibit a vast diversity of PTMs [reviewed in detail in (Kummer & Heneka, 2014)], including oxidation, phosphorylation, glycosylations, pyroglutamylation, as well as the formation of nitric-oxide altered nitrated Aβ and dityrosine-coupled Aβ. Furthermore, the Aβ peptide exhibits non-enzymatic isomerization at asparagine and aspartate, as well as racemization at aspartyl and seryl residues. Of these PTMs, pyroglutamylation is the most commonly detected in brain (Kummer & Heneka, 2014).

In parallel, with this highly MS-driven discovery of diversity in Aβ proteoforms (including PTMs), it was demonstrated that individual peptides aggregate differently and possibly form structurally distinct aggregates. With this, it became apparent that fundamental Aβ centric AD research cannot remain focused purely on the major Aβ peptides (Aβ1-42 and Aβ1-40). At the same time, however, while the notion of this peptide diversity remains in the background, the focus of the Aβ centric AD research has gradually shifted away from both individual and fully aggregated Aβ fibrils in Aβ plaques, to highly diversified intermediate assemblies such as Aβ oligomers and protofibrils. Assuming, that Aβ and its aggregation are indeed at the core of the onset of AD pathogenesis, this presents researchers with a challenging task: characterizing the molecular constituents that are likely highly diversified, both when it comes to the peptide backbone and their modifications, but also to the structural higher order assembly (oligomers).

4 | Aβ AGGREGATION AND BROAD CHARACTERIZATION OF THE ASSEMBLIES

A common feature of all amyloidogenic proteins is the presence of a hydrophobic amino acid component. Indeed, Aβ peptides have two such regions, the previously mentioned C-terminus (commonly considered aa 38-42), and the mid-region domain, the so-called KLVFF (aa 16-20) motif. These features make the peptide more or less prone to aggregate. A widely accepted generic mechanism for this process is the folding of the C-terminal region onto the mid-region of the peptide to generate a β-hairpin (Jarrett et al., 1993; Serpell, 2000; Tjernberg et al., 1999). This folding depends on the aromatic-stacking interaction of the two phenylalanine residues present in the KLVFF motif. These aromatic moieties stabilize intra-molecularly within a single peptide (but also the intermolecular interactions in larger assemblies) (Cukalevski et al., 2012; Gazit, 2005; Rambaran & Serpell, 2008). Furthermore, the salt bridge between the anionic carboxylate of the aspartic acid residue (aa 23), and cationic ammonium lysine residue (aa 28) also help sustain the loop region (Berhanu & Hansmann, 2012; Larini & Shea, 2012). Subsequently, aggregation of multiple β-hairpins, along with conformational rearrangements and formation of hydrogen bonds between adjacent strands, results in the formation of higher order assemblies (Figure 1d) (Hoyer et al., 2008; Schmidt et al., 2009; Serpell, 2000; Tjernberg et al., 1999). Some of the earlier mentioned mutations in APP affect this hydrophobicity driving amyloid formation.

To date, many different types of synthetic and natural Aβ aggregates have been reported (Benilova et al., 2012). In a simplified view, the key components of the Aβ aggregation cascade include the initial aggregation of the peptide into either dimers or other lower and higher order n-mers. Eventually, these form protofibrils that later combine to generate fibrils (Figure 1d); for the detailed review of identified aggregate species and their interconnections see Benilova et al. (Benilova et al., 2012). Furthermore, there is the commonly accepted concept of “soluble” and “insoluble” Aβ aggregates. The soluble Aβ assemblies comprise saline extracted physiological Aβ aggregates. These are Aβ species that do not precipitate during high-speed centrifugation when Tris-buffered saline is used as an extraction solvent. Recently, this description was expanded to include extracts of widely termed “soluble Aβ” with the aid of detergent, particularly triton or SDS. On the other hand, “insoluble” species comprise material obtained from formic acid (FA) treated pellets.

Extraction of the soluble and insoluble Aβ assemblies is often performed sequentially, and yields material for subsequent analysis, for instance using antibodies. For a long period of time, this analysis has been primarily monomer centric, but analysis of Aβ oligomers is becoming more frequent (Englund et al., 2007; Lee et al., 2006).

Indeed, such an approach demonstrated that water-soluble and triton-soluble, but not FA-soluble, Aβ-containing fractions are elevated in AD compared with non-demented cases (McLean et al., 1999). Similarly, these approaches allowed for pioneering identification of now well-recognized forms of Aβ aggregates, including Aβ dimers (Jin et al., 2011) and Aβ protofibrils (Walsh et al., 1997, 1999), but also intermediate oligomer species (Chimon et al., 2007). While the sequential extraction is rather unified across the AD research, to date there is little agreement as to which chemical and physical properties, associated with the long list of identified Aβ oligomers (both synthetic and native) are physiologically relevant in human AD (Benilova et al., 2012).

The amyloidogenic nature of Aβ does of course make it problematic to evaluate the efficiency of different extractions. It must therefore be taken into consideration that measurements are biased, and caution should be taken when comparing results using different protocols or even when the same protocol has been utilized in different laboratories. For example, the precise conditions during sequential extractions will affect cutoff levels and with a continuum of physicochemical properties of the sample compounds thus produce fractions which may vary in content between experiments.

In addition to reports of a diversity in higher order soluble Aβ aggregates, and their suggested distinct aggregation pathways [for a detailed review see (Benilova et al., 2012)], multiple mechanisms as to the origin and nature of the soluble Aβ aggregate driven activity have been proposed; for a review see (Haass & Selkoe, 2007). This brings forth the question, are the supposedly distinct forms of oligomers truly different from one another and, if so, do they indeed exhibit various degrees of toxicity? Without undermining any previous work regarding Aβ aggregates, it is rather safe to say that the answer to this question might not be as straight-forward as one might think.

5 | DEFINING SOLUBLE Aβ AGGREGATES IS AN ANALYTICALLY CHALLENGING TASK PRONE TO ERRORS

The majority of modern Aβ oligomer research relies on two intuitively correct assumptions that, unless proven otherwise, oligomers are soluble and prefibrillar, and that their properties are associated with their size measured primarily through western blot (WB) in combination with SEC. While such categorization does help in characterizing some of the physical properties of Aβ aggregates along the amyloid cascade hypothesis, a significant line of emerging research points away from such a simplified approach. Multiple aspects, not limited to Aβ aggregate isolation, preparation, and actual analysis, can significantly affect the properties of the Aβ assemblies that are investigated.

Already in the general survey of the reported Aβ assemblies, one quickly notices that some of the oligomeric species are reported exclusively, either in synthetic preparation, or in biological samples (Benilova et al., 2012). The natural biological variability and levels could underlie these discrepancies. Alternatively, differences in amyloidogenic properties of naturally occurring oligomers, and those made in synthetic preparation could exist. Indeed, when Aβ seeding paradigms were used to assess the ability of exogenous Aβ to induce Aβ pathology, earlier studies have suggested that synthetic Aβ does not efficiently induce amyloid formation in vivo (Meyer-Luehmann et al., 2006). However, more recent reports have demonstrated that synthetic Aβ might indeed possess amyloid-forming properties upon introduction (Stohr et al., 2012). Still, the purified Aβ was reported to be more efficient at inducing amyloid formation than the synthetic one. One side this does suggests that the synthetic Aβ aggregates might not truly reflect native ones, and their Aβ-pathology inducing properties. Still, one cannot exclude the possibility that additional factors (except Aβ itself), that are extracted from the brain tissue, and that are not present in the preparation of synthetic Aβ, do contribute to the Aβ seeding properties.

Then comes the concern of a generic preparation/isolation of the Aβ assemblies and how they are characterized across the different laboratories. Multiple aspects, not limited to concentration, temperature, pH, salt, other proteins, and lipids, are all known to affect the way and rate Aβ peptides aggregate, and how stable they are (Zapadka et al., 2017). For instance, just by using the earlier mentioned SDS, it is possible to induce artificial oligomerization of Aβ (Bitan et al., 2005). There are many other molecules with similar properties, and hence any work with Aβ requires either preparation/isolation standardization or, to achieve even higher reproducibility between laboratories, a precise characterization of the sample.

Regardless of the approach used, what is typically measured in the end to determine the type of Aβ aggregates one has is a band in a WB analysis. Such bands confirm the size of the Aβ assembly. In the case of single monopeptides (*e.g.*, only Aβ1-42 preparation), or controlled animal research, this might be sufficient to keep the procedures "standardized." However, complex human samples contain a wide array of Aβ peptide proteoforms, which renders this approach less than optimal.

If the preparation and analytical approaches do indeed affect what Aβ assemblies we see, and their potentially distinct conformation, one needs also to assess both the structural properties of these aggregates (including conformation) and the true molecular character of such species (composition). Otherwise, there is no way to actually know if the observed WB bands truly represent Aβ assemblies or, as repeatedly demonstrated, larger APP fragments. Indeed, incorrect assumptions about the nature of proposed oligomeric species have been made before. Lesné et al. proposed the existence of a soluble ~56 kDa Aβ species after analyzing transgenic mouse brains using SEC and WB (Lesné et al., 2006). A follow-up study in human brain was also carried out supporting a role of this species in AD (Lesné et al., 2013). Later, it turned out that a likely explanation for the ~56 kDa Aβ species (and other species with mass ≥15 kDa) was instead N-truncated forms of sAPP-α (Grant et al., 2019). Many such N-terminally extended (NTE)-Aβ have been reported with the help of MS (discussed below), and likely underlie many of the "oligomeric species" reported throughout the literature.

In a highly complex biological setting, the assumption that Aβ assemblies are homogeneous (*i.e.*, built up of the same Aβ peptide) is rather oversimplified as well. Not only has it been demonstrated that native Aβ assemblies are heterogeneous when it comes to Aβ peptide composition but also that Aβ can form aggregates and complexes with other biomolecules including proteins and lipids. Again, MS can receive a lot of credit for these discoveries.

This brings up the next aspect of studying Aβ assemblies, which is their stability during sample preparation. Again, the use of SDS-PAGE analysis is a great example. When applied to generally termed Aβ monomers, Aβ oligomers, and Aβ fibrils, the preparation yields monomers, dimers, trimers, and tetramers in all three types of complexes, respectively (Hepler et al., 2006). This demonstrates that many of the Aβ assemblies, including the "insoluble" Aβ fibrils are unstable.

Considering the fact that not only Aβ monomers, but also higher order aggregates, are degraded under physiological conditions to maintain homeostasis, this also raises the question of whether some of the oligomers are "more stable" than others? Such stability could originate from the covalent bonding between Aβ (but also potentially with other proteins). Indeed, covalently bound species exist (Brinkmalm et al., 2019), and may be present in a continuous dynamic equilibrium. If this is the case, this equilibrium would vary in presence of different peptides, their concentration, as well as multiple other factors mentioned above. Therefore, in the context of in vivo assays of Aβ assemblies, it might be hard to delineate what component of the preparation of synthetic or native assemblies causes the observed effect. Likewise, the various toxic effects of Aβ assemblies might not stem simply from the amount of the aggregates, but also their heterogeneity and possible PTMs (which unless induced will be absent in synthetic preparations). In this context, the field of AD research, particularly the more clinically related, has fallen into the dangerous notion that all soluble Aβ assemblies are toxic, and that they are all the same.

In order to advance the characterization of Aβ assemblies, approaches that enable an analysis of Aβ species in their "native" form need to be established. Here, exploring PTMs that might occur under physiological conditions might provide an effective approach to study Aβ assemblies. For instance, phosphorylation, including that of serine (aa 8), contributes to the formation of less compact Aβ conformations (Rezaei-Ghaleh et al., 2016a; Rezaei-Ghaleh, Kumar, et al., 2016). Another PTM, the pyroglutamate modification of the N-terminus, has been suggested to accelerate aggregation (Dammers et al., 2017; Schlenzig et al., 2009). Alternatively, means of stabilization of the Aβ assemblies have to be developed. Molecular stability is usually achieved through cross-linking (and the formation of covalent bonds) between adjacent species. Such cross-linking can be induced by chemical reactions initiated through, for example, heat, pressure, change in pH, or irradiation. Indeed, generation of covalent bonds in photo-cross-linking (*e.g.*, using photo-induced cross-linking of unmodified proteins) is one of the promising approaches (Bitan et al., 2001, 2003; Fancy & Kodadek, 1999).

6 | CONFORMATIONAL POLYMORPHISM OF Aβ ASSEMBLIES IS A FACT THAT SHOULD NOT BE OVERLOOKED

In a simple world, a common mechanism of pathogenesis irrespective of the structure of the aggregates, or at least relying on the diversity of Aβ oligomers as defined by their size and solubility, would simplify AD research tremendously. Indeed, long sought-out conformational antibodies targeting generic epitopes present in either prefibrillar oligomers or fibrils have been reported (Kayed et al., 2003, 2007, 2010; Kayed & Glabe, 2006; Lesné et al., 2006), and these hold great promise. Sadly, such approaches might not be enough. The recently growing area of studying conformational polymorphism (Fändrich et al., 2018; Meyer-Luehmann et al., 2006; Toyama & Weissman, 2011), *i.e.*, diversity in conformation of Aβ assemblies (including those of both the same size and structure), might instead be the key to delineating the Aβ diversity and toxicity.

Hints of such diversity in Aβ assemblies already exist in the Aβ plaque pathology. In sAD, Aβ plaque pathology manifests itself primarily in cored, "congophilic" deposits, and diffuse plaques (Howie & Brewer, 2009). Plaques (primarily diffuse type) have also been found in non-demented subjects (CU-AP patients) (Dickson et al., 1992). The debate of whether the diffuse and cored plaques are the result of different degrees of Aβ maturation (be it fibril conformational changes of biochemical changes such as PTMs), or are formed through distinctly separate mechanisms, and whether some rather than other are more or less representative of toxicity, is still ongoing (Dickson et al., 1992; Dickson & Vickers, 2001; Lord et al., 2011; Masliah et al., 1990; Philipson et al., 2012). However, recent work from Rijal Upadhaya et al. (for Aβ plaque pathology) and Gerth et al. (for vascular amyloid), investigating preclinical as well as symptomatic AD has demonstrated that advancement of the disease pathology has associated with biochemical staging that involves first Aβ deposition, subsequent Aβ pyroglutamate formation, and finally Aβ phosphorylation (Gerth et al., 2018; Rijal Upadhaya et al., 2012). Therefore, it is clear, the end-stage Aβ assemblies are morphologically and biochemically different. Given the fact that Aβ plaques in fAD are even more structurally diverse (Rasmussen et al., 2017), speculation on distinct aggregation trajectories might not be far from the truth.

Recent studies of higher order Aβ aggregates demonstrated a high degree of conformational variation among oligomers, protofibrils, fibrils, and even plaques (Fändrich et al., 2018; Hammarström, 2019; Rasmussen et al., 2017). This is proposed to originate from the differences in folding of individual peptides and oligomers, and, respectively, the assembly of individual protofibrils into fibrils and plaques (introduced above) (Tywoniuk et al., 2018).

Structural spectroscopy based on luminescent conjugated oligothiophene (LCO) probes, has been used to demonstrate age-dependent changes in conformational polymorphism within individual plaques, conformation-specific properties of prions, and, most recently, variability in Aβ amyloid aggregate structures

between plaques of AD subtypes (*e.g.*, fAD and sAD) (Klingstedt, Blechschmidt, et al., 2013; Klingstedt, Shirani, et al., 2013; Magnusson et al., 2014; Nyström et al., 2013; Rasmussen et al., 2017). Similarly, cryo-EM and solid-state nuclear magnetic resonance spectroscopy studies have indeed visualized folding polymorphism not only in-between different peptide aggregates, for example, Aβ1-40 and Aβ1-42, respectively, but also that the same peptide, for example, Aβ1-42, can fold differently, resulting in multiple structural models of Aβ fibrils (Gremer et al., 2017; Lu et al., 2013; Saido & Leissring, 2012; Schmidt et al., 2009; Zou et al., 2013). In addition to synthetic in vitro studies, such as analysis of extracts from clinical subtypes of AD, have also demonstrated a high degree of conformational polymorphism (Qiang et al., 2017).

This high degree of conformational polymorphism is, however, not only unique for higher order aggregates; instead, it has been proposed to be encoded already in the early stages of aggregation, including species as small as dimers (Wei et al., 2010). Subsequent growth of these occurs through highly heterogeneous interaction associated with conformational changes that are much more complex than parallel or antiparallel orientation present in fibrils (Ono et al., 2009; Schmidt et al., 2009; Zou et al., 2013).

In the context of biological activity, it is therefore not surprising that small changes in the relative amounts of, for instance, Aβ1-40 and Aβ1-42 (Kuperstein et al., 2010), or presence of PTMs, including pyroglutamation (Schlenzig et al., 2009), or phosphorylation (Kumar et al., 2011), dramatically affect both aggregation and neurotoxicity of Aβ. On a larger scale, this conformational polymorphism of Aβ assemblies poses tremendous challenges to the development of any therapeutics targeting higher order aggregates (*e.g.*, dimers or oligomers), as these then face issues "fitting" the shape of the amyloid (Fändrich et al., 2018). Furthermore, it poses problems related to the detection and visualization of amyloid structures for diagnostic purposes (Hammarström, 2019).

7 | MS HAS DRIVEN A LARGE PART OF THE Aβ RESEARCH, AND THE TECHNIQUE HAS MUCH MORE TO GIVE

MS has driven a large portion of the research underlying the current understanding of Aβ centric research in AD. From aiding first identification of the Aβ peptide sequence, through demonstration of diversity in Aβ peptide proteoforms in brain tissue and bodily fluids, to demonstration of structural and conformational diversity among Aβ aggregates. With recent developments, the technique has been used directly on the tissue to detect Aβ peptide diversity; in extracts, it demonstrates sites of interactions in Aβ dimers and diversity in dimer species; and finally it holds promise to allow for monitoring of different oligomers' sizes and their interconversion (Bleiholder & Bowers, 2017; Bleiholder et al., 2011). Here we outline the historical contribution of MS in Aβ centric AD research, focusing on human brain tissue, CSF, and plasma analysis.

8 | WORK ON BRAIN EXTRACTS CHARACTERIZES Aβ PROTEOFORM DIVERSITY

The first chemical characterization of Aβ was performed in 1983 by Allsop et al., who determined its general amino acid content by isolating plaque cores from human brain tissue (Allsop et al., 1983). In 1984, Glenner & Wong isolated the Aβ protein from cerebrovascular amyloid in AD and DS brains and sequenced the first 24 N-terminal aa of Aβ (Glenner & Wong, 1984a, 1984b). The following year Masters et al. isolated from AD and DS plaque cores, did the first assessment of solubility of Aβ in a variety of conditions, and determined additional N-terminal amino acids (Masters et al., 1985). Importantly in this work they also observed an N-terminal heterogeneity, and the presence of larger (8 kDa and 16 kDa) Aβ species. Finally, a few years later Mori et al. demonstrated the full sequence of Aβ1-40 and Aβ1-43, by employing plasma desorption mass spectrometry (PDMS) on enzymatically LysC-digested high-performance liquid chromatography (HPLC)-purified fractions. Additionally, this work proved the unique capability of MS to identify PTMs in Aβ, identifying pyroglutamate formation as the result of dehydration at the N-terminal glutamic acid (Mori et al., 1992).

Subsequent matrix-assisted laser desorption/ionization (MALDI)-MS analyses corroborating the finding by Mori et al. were made by Roher et al. in 1993 (Roher, Lowenson, Clarke, Wolkow, et al., 1993). This work investigated potential post-translational alterations of Aβ aspartic acids, suggesting that such PTMs could alter the peptide conformation and in turn increase aggregation propensity. Using PDMS, Roher also reported Aβ1-42, followed by Aβ1-40, to be the major component of both leptomeningeal and parenchymal blood vessels (*i.e.*, CAA) in purified AD brain extracts (Roher, Lowenson, Clarke, Woods, et al., 1993). In this work, the authors also suggested such vascular amyloid to be "younger" than amyloid plaques, given the less isomerized and racemized aspartyl residues. About the same time, Miller et al. reported eleven N-truncated endogenous Aβx-42 peptides from brain tissue analyzed using MALDI-MS (Miller et al., 1993). The first electrospray ionization (ESI)-MS analysis of intact Aβ from brain homogeneates was performed in 1994 by Näslund et al. (Näslund et al., 1994). This analysis included multiple sAD cases, two fAD cases with Swedish and, London mutation, respectively, and several non-demented elderly. This allowed for the identification of 10 different N-terminally truncated forms all ending at aa 40 and 42 (Aβx-40 and Aβx-42). Interestingly, the predominant Aβ variant in sAD was the Aβ1-40, whereas the longer, and generally considered more toxic, Aβ1-42 was identified as the main species in the non-demented controls. The difference in the ratio of the Aβ1-40/Aβ-42 was reported to be 10-times greater in sAD as compared with non-demented controls. A few years later Wang et al. obtained similar results in antibody-based enzyme-linked immunosorbent assay (ELISA) measurements of Aβ1-40 and Aβ-42 in soluble and insoluble fractions from sAD and CU-AP patients (Wang et al., 1999a). Here, 10-fold higher levels of insoluble Aβ1-40 (and only 2-fold higher insoluble Aβ1-42) were

found present in brains of AD patients as compared with CU-AP. On the other hand, the soluble Aβ1-40 and Aβ1-42, as a fraction of total Aβ, were higher in CU-AP.

Alongside the direct measurements of whole-brain extracts, the first approaches for immunoprecipitation (IP)-based Aβ enrichment prior to MS analysis were developed. Initially demonstrated in cells in 1996 by Wang et al. (Wang et al., 1996), these approaches enabled study of γ-secretase activity in vitro (using MALDI-MS) (Murphy et al., 1999). In 2010, Portelius et al. applied IP-MS (both MALDI and ESI) to investigate whole-brain extracts from cerebellum, cortex, and hippocampus of sAD, fAD cases (with Swedish and *PSEN1* mutations), and non-demented controls (Portelius, Bogdanovic, et al., 2010). They observed multiple C- and N-terminally truncated Aβ proteoforms, including those with pyroglutamate-modified N-terminus. Aβ1-42, Aβ4-42, and Aβ1-40 appeared to be dominant species. Broadly, no prominent differences in Aβ proteoform coverage patterns between sAD and fAD were present (the absolute amounts were not probed), underscoring the similarity in the amyloid pathology of these two disease entities.

Two years later Moore et al. applied a similar approach to look at sequential extracts from sAD, CU-AP, and non-demented control brain (Moore et al., 2012). Again, they observed multiple N-terminally truncated Aβ peptides, which appeared slightly elevated in sAD. The overall Aβ proteoform profile of CU-AP was similar to sAD (although differing between extracts) supporting the view that CU-AP is a preclinical stage of sAD. Similar, elevated N-terminal truncations' levels in sAD as compared with CU-AP was later reported by Portelius et al., although in single extracts (Portelius et al., 2015). In line with previous reports, Gkantsiou et al. using MALDI-MS observed an increase in the portion of Aβx-40 peptides and a corresponding decrease in the portion of Aβx-42 in AD compared with CU-AP (in this issue: Gkanatsiou et al). Furthermore, pGlu Aβ3-40 and pGlu Aβ3-42 both had higher relative portions in AD (Fukumoto et al., 1996; Harigaya et al., 2000; Kuo et al., 1997; Michno, Nystrom, et al., 2019) (in this issue: Gkanatsiou et al).

Together, the overall work on brain extracts has demonstrated highly complex, yet similar Aβ proteoform profiles in all sAD, fAD, and CU-AP patients. Instead of looking for unique peptides or quantifying the overall Aβ load, this work suggests that it might be the differences in the overall levels of the different Aβ proteoforms that underlie the disease and its progression, at least when it comes to the load of Aβ assemblies and plaques.

The basic assumption driving the overall work of Aβ reported in whole-brain extracts is that the Aβ plaques represent the insoluble Aβ species. The origin of the soluble species cannot, however, be as easily presumed. Soluble oligomer species might come from plaques or plaque vicinity, but also from "plaque free" areas. In this context, looking at the Aβ peptide composition in individual plaques might be the key to untangle the complicated story of the Aβ proteoforms and their role in aggregation and plaque formation. Indeed, while the Aβ1-42 is considered the primary toxic peptide in the context of AD, and is likely the seed of the later formed fully-grown Aβ plaques as present in symptomatic AD cases (Aβ plaques rich in both a diverse Aβ proteoforms and in PTMs); this peptide appears to be a dominant species also in CU-AP and non-demented controls. So what do plaques actually consist of?

9 | ADVANCEMENTS IN MS ENABLE Aβ PROTEOFORM ANALYSIS ON A SINGLE PLAQUE LEVEL

Conventional assessment of the Aβ pathology in the brain is performed through histochemical analysis of Aβ deposits, either using different anti-Aβ antibodies or histological amyloid probes, such as Congo Red (CR) and Thioflavin. Indeed, deposits are broadly divided into dense cored plaques, often referred to as "congophilic" based on their detection with CR stain, and diffuse plaques, which cannot be visualized with CR (Howie & Brewer, 2009). While such division provides a general indication of the aggregation state Aβ assemblies present in the plaques, epitope-specific approaches based on antibodies can instead be used, in order to demonstrate the presence of common Aβ proteoforms in Aβ plaques.

The initial assessments of Aβ peptides on a single Aβ plaque level (using histological techniques) were demonstrated in the mid-90s by Iwatsubo et al. (Iwatsubo et al., 1994, 1995), Lemere et al. (Lemere, Blusztajn, et al., 1996), and Mann et al. (Mann & Iwatsubo, 1996; Mann, Iwatsubo, Cairns, et al., 1996; Mann, Iwatsubo, Ihara, et al., 1996; Mann et al., 1996c, 1997). These works followed age-related changes in Aβ pathology in DS patients, and suggested that in brains from younger DS patients, the Aβx-42 were the dominant proteoforms and that presence of Aβx-40 plaque cores increased with age (Iwatsubo et al., 1995). Similar results were concurrently reported for sAD (Iwatsubo et al., 1996), where the diffuse plaques were Aβx-42 immunoreactive, but Aβx-40 negative.

Assessment of fAD cases, including subjects with APP717 mutations (London and Japanese) (Mann, Iwatsubo, Ihara, et al., 1996), and PSEN mutations (Lemere, Lopera, et al., 1996; Mann, Iwatsubo, Cairns, et al., 1996), which all affect the γ-secretase activity, displayed predominantly Aβx-42 immunoreactive plaques. The fAD patients with Swedish mutation displayed relatively increased Aβx-40 immunoreactivity, which still, however, appeared secondary to Aβx-42 (Mann, Iwatsubo, Ihara, et al., 1996). Interestingly, CAA, which is common in sAD (Iwatsubo et al., 1995; Serrano-Pozo et al., 2011) but also in the here mentioned fAD cases (Swedish, Japanese, and London) and in the vascular dementia (Dutch type), was reported to be highly Aβx-40 immunoreactive (Mann, Iwatsubo, Ihara, et al., 1996).

Therefore, a complicating factor in the earlier mentioned differences in levels of different Aβ proteoforms in whole-brain extracts reviewed above could be tied not only to the solubility of the different Aβ assemblies (as suggested by differences in sequential extracts), but could additionally stem also from the high frequency of CAA comorbidity.

Regardless of the context of Aβ proteoforms and perceived toxicity, it is noteworthy that already the early work demonstrated

that the increased deposition of Aβx-40 (but not Aβx-42) associated with core formation, was also demonstrated to be dependent on the gene dosage of the apolipoprotein E (*APOE)* ε4 allele, a major risk gene for sAD (Mann et al., 1997) (the role of *APOE* in Aβ proteoform deposition is beyond the scope of this review). Similarly, an increase in Aβx-40 content was also linked to not only more developed Aβ pathology but also an increase in synaptic loss (Lue et al., 1999), a result recently confirmed using different MS techniques (Gkanatsiou et al., 2019).

The high content of Aβx-40 in CAA on the other hand might be associated with vascular Aβ clearance that gradually becomes impaired. Following the initial seeding of Aβx-42 in the vessel walls the otherwise more soluble and much more abundant Aβx-40 does deposit (Weller et al., 1998, 2000). This deposition of Aβx-40 peptides has been suggested to underlie spontaneous cerebral and lobar hemorrhages (Gibbons & Dzau, 1994; Kumar-Singh, 2008; Weller et al., 1998). Indeed, subjects with fAD mutations that lead to an increased total Aβ, but not Aβ1-42/Aβ1-40 ratio, display a higher risk of CAA-associated hemorrhage (Grabowski et al., 2001; Levy et al., 1990; Nilsberth et al., 2001). The Aβ1-40 fibrils have been reported to be more rigid than the Aβ1-42 (Dong et al., 2016). Finally, a strong localization of quadro-formylthiophene acetic acid (q-FTAA) (a fluorescent amyloid dye indicating high maturity and density of fibrils) is reported for highly Aβx-40-positive parenchymal deposits, and even more so CAA (Michno, Nystrom, et al., 2019; Rasmussen et al., 2017).

Just like ELISA or WB for assessment of Aβ in extract, antibody-based analysis of Aβ plaque pathology suffers the general limitations of antibody-based approaches (*e.g.*, affinity, issues caused by PTMs, tissue penetration, and multiplexing), and also the challenges associated with their targets' conformational flexibility. Therefore, alternative approaches that offer better resolution and accuracy, in terms of Aβ polymorphism and Aβ proteoform content are warranted.

The issue of conformational diversity within Aβ deposits, has been to some extent addressed by the development of highly sensitive and conformation-specific electro-optically active chromophores, which enable detection of polymorphic amyloid structures (Leclerc, 2000). Particularly promising are luminescent conjugated oligothiophene probes (LCO), which have been used to demonstrate age-dependent changes in conformational polymorphism within individual plaques (although in mice), conformation-specific properties of prions and, most recently, variability in Aβ amyloid aggregate structures between plaques of AD subtypes (*e.g.*, fAD, sAD, and CU-AP) (Klingstedt, Blechschmidt, et al., 2013; Klingstedt, Shirani, et al., 2013; Magnusson et al., 2014; Michno, Nystrom, et al., 2019; Nyström et al., 2013; Rasmussen et al., 2017). Still, while such probes can be multiplexed with anti-Aβ antibodies (for monomers or potentially even oligomers), the degree of multiplexing is limited as there is only a finite number of fluorescent channels that can be combined without introducing the risk of "bleed through" and hence false positive signal. This makes it hard to determine the proteoforms underlying the Aβ plaque heterogeneity at a similar level as MS-based analysis of the brain extracts. An interesting approach to explore in this context would be the use of the antibody-mass spectrometry combination, referred to as mass cytometry imaging (Angelo et al., 2014; Giesen et al., 2014). Here, antibodies are conjugated to rare-earth metals rather than fluorophores, and detection is performed with help of a mass spectrometer. This approach allows for the visualization of tens of markers simultaneously.

When it comes to more plaque-specific analysis of Aβ proteoforms that are also direct (detect the Aβ species present by measuring their levels, not by measuring the binding of antibodies which is an indirect approach), two MS-driven approaches gained focus over the last few years, laser microdissection and imaging MS (IMS). Laser microdissection enables isolation/dissection of microscopic objects of interest with the help of laser and can be used for subsequent MS experiments. This approach allows for the isolation of specific types of Aβ aggregates, such as different types of Aβ plaques or CAA, but requires prior visualization of the target (*e.g.*, using antibodies). IMS, on the other hand, is a powerful way to approach concurrent probing of chemical distribution in complex biological tissue samples, without the need for such visualization (Caprioli et al., 1997; Hanrieder et al., 2013; McDonnell & Heeren, 2007; Michno et al., 2019b). Furthermore, the approach requires minimal sample preparation and treatment.

Until recently, the application of IMS on human tissue was only possible for analysis of the general Aβ peptide signature in the brain tissue rather than on a single plaque level (Philipson et al., 2012). Still, already at the point of initial application, IMS was able to demonstrate the presence of both N- and C-terminally truncated Aβ proteoforms in the brain tissue of patients with the Arctic mutation. Following improvements in analysis methods as well as developments in IMS instrumentation, the first application of IMS for analysis of individual Aβ inclusions in human tissue was demonstrated (Kakuda et al., 2017). The authors were able to identify a wide range of Aβ proteoforms that were both N- and C-terminally (including pGlu Aβ3-x peptides) truncated in broadly classified senile plaques and leptomeningeal CAA.

On the other hand, a more Aβ pathology (Aβ plaque subtype) specific application of laser microdissection was demonstrated on antibody-detected, Lys-C digested deposits from CU-AP and sAD subjects. This work showed that the diffuse plaques are predominantly Aβx-42 positive, while the more aggregated Aβ plaques as well as CAA, contained predominantly Aβx-40 proteoforms (Güntert et al., 2006). Additionally, this study suggested that N-terminally pyroglutamated species, pGlu Aβ3-16 (termination at aa 16 because of digestion) were increased in more aggregated deposits.

Recently, a similar cohort was also examined with help of laser microdissection. Here, the authors collected both the conformational information available by using LCOs (rather than antibodies), and the precise proteoform information by using IP for purification, and subsequent MS analysis of undigested, endogenous, peptides. Besides confirming the earlier laser microdissection-based work (Güntert et al., 2006), the study identified Aβ4-42, rather than Aβ1-42, as the major species in both sAD and CU-AP plaques (both cored and diffuse) (Michno, Nystrom, et al., 2019). Aβ1-40 was indeed present

in deposits that contained a highly aggregated center, as well as in CAA. Importantly, thanks to the use of LCOs, the authors were able to demonstrate that diffuse plaques in sAD and CU-AP differed when it comes to the aggregation state (though visually similar). These aggregation differences appeared to be associated with an increased pyroglutamation of Aβ ending at "aa 42," suggesting hydrophobic functionalization of diffuse plaques which later facilitated Aβ1-40 deposition. This study further complemented the use of laser microdissection with IMS and allowed for precise visualization that Aβ1-40 is indeed dominating at the core of the plaques. Additionally, it demonstrated that the N-terminally truncated Aβx-42 and Aβx-40 peptides followed similar deposition patterns as their full-length counterparts (Aβ1-42 respective Aβ1-40) on a single Aβ plaque level.

Together, these works demonstrate that laser microdissection and IMS enable probing the Aβ proteoforms in Aβ inclusions across different brain regions, and structural morphotypes at a resolution similar to that of histochemical analysis (*e.g.*, with antibodies), but at the specificity obtainable only through direct measurements by MS. Furthermore, they hold the potential of investigating general biochemical microenvironments associated with individual Aβ inclusions, as demonstrated for phospholipids and sphingolipids in transgenic AD mouse models (Kaya et al., 2017; Michno et al., 2018, 2019c). Still, the question remains whether these approaches will aid in the characterization of lower order Aβ assemblies, such as soluble Aβ. And does the work in postmortem tissue truly reflect the presumed inverse Aβ profile present in CSF (and recently also blood/plasma) Aβ measurements?

10 | Aβ AS A FLUID BIOMARKER—MULTIPLE Aβ PROTEOFORMS BUT A SINGLE Aβ1-40/Aβ1-42 RATIO

Aβ was not directly detected in CSF until the early 1990's when Haass et al. found that Aβ was produced and secreted during normal cell metabolism (Haass et al., 1992), and Seubert et al. (Seubert et al., 1992) and Shoji et al. (Shoji et al., 1992) used affinity chromatography with follow-up sequencing and IP-WB to show that Aβ was present in CSF from individuals with AD, but also in cognitively normal subjects. In 1993, Vigo-Pelfrey et al. (Vigo-Pelfrey et al., 1993) measured Aβ in CSF using IP with an affinity column and subsequent MALDI-MS analysis of collected fractions; several variants of Aβ peptides were found, the longest being Aβ1-40. Furthermore, the authors reported the presence of larger species (dimers and trimers), whose nature was, however, not confirmed. By 2000, several studies had come to the conclusion that the CSF concentration of Aβ ending at aa 42 was lower in AD than in control subjects, while total Aβ was unchanged (Boss, 2000). However, the first attempts of actual quantification of Aβ using surface-enhanced laser desorption/ionization-MS, in a study of AD and control subjects published in 2004 by Maddalena et al., reported a general decrease in Aβ1-38 in AD (Maddalena et al., 2004). This study also revealed Aβ1-38 and Aβ1-40 as the major Aβ proteoforms in CSF.

Subsequently, more details have emerged with refined methods of sample purification and increasingly more sensitive instruments. In 2006 and following years, Portelius et al. and others published several articles expanding the number of Aβ peptides observed in CSF to well over 100 variants (Brinkmalm et al., 2012; Portelius et al., 2006; Rogeberg et al., 2015). These included non-canonical forms of NTE-Aβ (Portelius, Brinkmalm, et al., 2010). As discussed earlier, such species can give rise to an incorrect interpretation as Aβ aggregates (Grant et al., 2019). Alongside various Aβ primary structures, identification of additional proteoforms, such as peptides with multiple PTMs have been reported (Kummer & Heneka, 2014). As demonstrated by Halim et al., some of such PTMs are specific to certain Aβ proteoforms. For instance, an unusual O-glycosylation at Tyr10 of the Aβ sequence seems to be present only in shorter forms, Aβ1-15 through Aβ1-20 (Halim et al., 2011). This work also showed that NTE-Aβ could be O-glycosylated in a number of positions N-terminal of the BACE1 (β-secretase) cleavage site. Willem et al. described even longer NTE-Aβ cleaved by η-secretase, which cleaves between aa 504 and 505 of APP695 (Willem et al., 2015). Subsequent cleavage by A disintegrin and metalloproteinase domain-containing protein (ADAM10—α-secretase) or BACE1 creates the peptides APP/Aβ(−92 to 15) and APP/Aβ(−92 to −1), respectively (using Aβ numbering). Many of these are also present in cell media from induced pluripotent-derived neurons. Here, for instance, mutation-specific Aβ peptide patterns of PSEN mutations result in a relative increase in Aβ42 (and/or Aβ43) and a relative loss of shorter and more hydrophilic Aβ forms (Brownjohn et al., 2017; Koch et al., 2012; Mertens et al., 2013; Moore et al., 2015), suggesting γ-secretase dysfunction, which is reflected in the CSF from the same patients (Arber et al., 2020).

While the majority of these studies demonstrate a wide range of different Aβ proteoforms being present in CSF, to date it is still Aβ1-42 or the Aβ1-40/Aβ1-42 ratio that is used as diagnostic criteria for AD. Do the other Aβ species have no relevance for AD and are just non-specific by-products; or is it just the high complexity of this Aβ proteoform pool that holds back the discovery of potential keys to understanding the Aβ driven AD pathogenicity? Could it be so that various Aβ proteoforms reflect molecular processes that occur at different stages of the Aβ pathology progression? In order to answer these questions one needs to be able to perform robust quantification of the Aβ proteoforms present in the samples, and ultimately, study changes in their occurrence—their kinetics—during the disease progression.

Because of the amyloidogenic nature of Aβ, quantification with MS is generally even more problematic than with immunoassays. MS requires more concentrated and purer samples, which increases the risk of aggregation and/or loss of peptides to surfaces in the preparation and analytical systems. Interaction of analyte to surfaces is a critical issue for Aβ and very likely explains much of the interlaboratory variability even for immunoassays (Mattsson et al., 2013). Nevertheless, also after IP, samples are not particularly clean; there are numerous non-Aβ compounds that bind unspecifically to the beads, actually facilitates Aβ analysis by preventing Aβ-surface

interactions. In general, robust quantification using MS requires the addition of stable isotope-labeled peptides Aβ. However, handling of Aβ standards becomes even more problematic because of their higher purity compared with the samples. For MS analysis, the use of alkaline buffers is one way to tackle this problem. While carry-over of monomeric Aβ is extensive in nanoflow LC under acidic conditions and many types of dimeric Aβ species cannot even be detected, the problem is much reduced by employing alkaline conditions (Brinkmalm et al., 2019; Oe et al., 2006; Pannee et al., 2016). The potential problem with aggregation is more difficult to investigate. However, also here it appears that samples may suffer less from handling-induced aggregation than standards; Aβ in CSF has been shown to be less aggregation prone than in HEPES buffer (Padayachee et al., 2016).

The first reports of a robust quantitative approach came in 2006 when Oe et al. combined IP, liquid chromatography (LC) with alkaline mobile phases, and negative ion mode ESI-MS to investigate Aβ1-40 and Aβ1-42 in CSF, and correlated these with ELISA measurements of the same peptides (Aβx-40 and Aβx-42)(Oe et al., 2006). Consistent with previous works they found decreased Aβ1-42 in AD subjects, while the Aβ1-40 was unchanged. It is noteworthy that the correlation of MS data with ELISA measurement, for Aβ1-40 and Aβ1-42, was found much stronger for Aβ1-42 peptide. This could suggest different anti-Aβ antibody binding efficiency, but could also have alternative causes such as PTMs, truncations, or even Aβ aggregation.

Further developments of quantitative MS approaches were done by Lame et al. (Lame et al., 2011) who used an antibody-free approach that relied instead on a solid phase extraction cleanup prior to LC-ESI-MS, to measure not only Aβ1-40 and Aβ1-42, but to also include Aβ1-38. By also using alkaline solvents, but instead relying on positive ion mode, they demonstrated that running MS analysis in positive ion mode did not result in any loss of detection precision or specificity compared with immune-based purification and negative ion mode analysis. Furthermore, operation in positive ion mode also improved fragment ion specificity. A similar approach was later used by Pannee et al. (Pannee et al., 2013) to measure Aβ1-38, Aβ1-40, and Aβ1-42, and demonstrated the ability of such an assay to differentiate between AD and non-demented subjects. Pannee et al. later performed a cross-validation study where the Aβ1-38, Aβ1-40, and Aβ1-42 measurements were compared with Aβ fibrils measurements using ^{18}F-flutemetamol positron emission tomography (PET) (Pannee et al., 2016). They demonstrated that the use of ratios (Aβ1-42/Aβ1-40 and Aβ1-42/Aβ1-38) significantly improved concordance with an area under the receiver operating characteristic curve (and thereby diagnostic ability) when dichotomized for positive or negative amyloid PET. On the basis of this work, the International Federation of Clinical Chemistry and Laboratory Medicine Working Group for CSF proteins (IFCC WG-CSF—http://www.ifcc.org/ifcc-scientific-division/sd-working-groups/csf-proteins-wg-csf/), and the Alzheimer's Association Global Biomarker Standardization Consortium developed MS-based reference methods for Aβ1-42 (Korecka et al., 2014; Leinenbach et al., 2014). These methods have been formally certified by the Joint Committee for Traceability in Laboratory Medicine (JCTLM database accession numbers C11RMP9 and C12RMP1), have been validated against amyloid PET, and a reference material for CSF Aβ42 was recently released (ERM-DA480/IFCC, ERM-DA481/IFCC, and ERM-DA482/IFCC) (Boulo et al., 2020).

Alongside stable isotope-labeled peptide "spike-in" approaches, an alternative method based on SILK has been demonstrated for Aβ peptide quantification and the study of Aβ turnover. This approach relies on an infusion of stable isotope-labeled amino acids (in the context of human work, most commonly $^{13}C_6$-leucine) to label newly synthesized protein directly in vivo (Paterson et al., 2019). These newly synthesized Aβ peptides can then be directly measured in both plasma and CSF, and besides enabling general Aβ quantification, could also provide insight into the Aβ peptide kinetics. This approach was recently applied for Aβ1-38, Aβ1-40, and Aβ1-42 proteoform kinetics, to demonstrate a general slow-down in Aβ turnover rate with age (Patterson et al., 2015). Here, Aβ1-38 and Aβ1-40 had similar kinetics regardless of the Aβ status (amyloid PET), while the kinetics of soluble Aβ1-42 was increased specifically in the Aβ positive individuals.

A similar SILK approach has also been used to study the diurnal pattern and effect of sleep on CSF Aβ kinetics. Looking at Aβ1-40 and Aβ1-42 diurnal pattern in subjects with and without amyloid deposition revealed an early age-associated loss of Aβ1-42 day/night levels, as well as a decline in Aβ1-42 over time (serial CSF sampling), in amyloid-negative individuals (Lucey et al., 2017). These changes were not present in amyloid-positive individuals, indicating that the production and clearance mechanism of Aβ1-42, associated with sleep–wake cycle are affected in subject with amyloid deposition. Aβ1-40 followed a different pattern, where there was an age-associated loss of Aβ1-40 day/night levels in both the amyloid positive and negative subjects (although significant only in positive). There was no decline in Aβ1-40 over time in the serial sampling in either of the groups. In another study looking at sleep deprivation in cognitively normal controls, it was found that sleep deprivation increased overnight levels of all Aβ1-38, Aβ1-40, and Aβ1-42 (Lucey et al., 2018). Finally, as previously suggested in cells, and recently verified in CSF by Liebsch et al, the Aβ34 appears to be a degradation intermediate of the BACE1 cleavage (Fluhrer et al., 2003; Liebsch et al., 2019; Shi et al., 2003). In this recent study, the authors used SILK to demonstrate the correlation of Aβ1-34 levels with the overall Aβ clearance rates in Aβ positive individuals, and a change in the Aβ1-34/Aβ1-42 ratio to reflect Aβ degradation and cortical deposition, together revealing a new potential marker for Aβ clearance in neurodegeneration.

Together, these data demonstrate the tremendous potential of SILK not only for quantification of Aβ proteoforms, but also for studying Aβ proteoform kinetics. They highlight the relevance of different Aβ peptides at various stages of Aβ pathogenesis, thereby verifying the need for precise characterization of Aβ peptide dynamics at different stages along with the AD progression.

11 | DETECTING Aβ PLASMA AND SERUM LEVELS—PREVIOUSLY UNATTAINABLE, NOW RIVALING CSF AND PET

AD diagnostics have for a long time been performed based on Aβ measurements in the CSF and through amyloid imaging using PET. These gold standard approaches are excellent tools for AD diagnostics. They are, however, invasive and expensive. Therefore, the development of sensitive and specific blood-based test for Aβ has for a long time been sought after. Such blood-based tests would be much more accessible, and could function as first-line tool for detecting pathophysiological changes present in AD.

In comparison to CSF measurements of Aβ, there are few MS studies conducted of Aβ in serum and plasma. The two main reasons are the close relationship between brain and CSF, and thereby lower concentration of Aβ in plasma as compared to CSF, as well as a much higher general protein concentration present in plasma as compared to CSF. Together, these make the MS analysis more challenging. Furthermore, the stability of Aβ in plasma and serum has been shown to be problematic since it is subject to cleavages by enzymes to a much higher extent than in CSF (Bibl et al., 2012; Portelius et al., 2017). Nevertheless, successful attempts have been made to analyze Aβ in plasma. Although plasma Aβ analysis using immunoassays was first reported in 1996 (Scheuner et al., 1996), the first publication using MS was in 2014 when Kaneko et al. used IP followed by MALDI-MS to detect more than 20 Aβ peptides including several NTE-Aβ (Kaneko et al., 2014). Shortly after Pannee et al. published data from IP combined with both MALDI-MS and LC-ESI-MS measurements (Pannee et al., 2014); demonstrating the presence of 11 Aβ peptides verified by MS/MS, including NTE-Aβ forms.

The driving force behind the attempts to analyze Aβ in plasma by MS was the lack of suitable plasma biomarkers for AD. Attempts to apply Aβ immunoassays to plasma have for a long time been inconclusive (Mayeux et al., 2003; Yaffe et al., 2011), similarly to the results from Pannee et al. However, in studies performed by Kaneko et al. (Kaneko et al., 2014) and later Nakamura et al. (Nakamura et al., 2018) Aβ1-42 plasma concentrations were significantly lower in AD compared with control subjects.

Recently Ovod et al. adapted the previously established SILK approach for measurements of Aβ1-38, Aβ1-40, and Aβ1-42 proteoform kinetics in plasma, and demonstrated that the overall stability of Aβ in plasma is very low with a half-life of 3 hr (compared to 9 hr in CSF) (Bateman et al., 2006; Ovod et al., 2017). They found that the turnover rate of Aβ1-38 is in general higher than that of Aβ1-40 and Aβ1-42. When looking at Aβ status, Aβ1-42 turnover kinetics was found to be higher in Aβ positive individuals. Furthermore, the overall levels of Aβ1-42 and Aβ1-42/Aβ1-40 were lower in Aβ positive subjects. A subsequent study by Schindler et al. (although without SILK), demonstrated a high correlation of plasma Aβ1-42/Aβ1-40 with Aβ PET (Schindler et al., 2019). Furthermore, they demonstrated that for PET-negative subjects, there was a 15-fold greater risk of conversion to PET-positive status in individuals with positive, as compared to negative, Aβ1-42/Aβ1-40 plasma status. The Aβ42/Aβ40 ratio is reduced by only 14%–20% in plasma, compared with 50% in CSF, with a greater overlap between Aβ-positive and -negative individuals, and the correlation with CSF is weak. This could be explained by the production of Aβ peptides in platelets and other non-cerebral tissue. Still, the concordant research findings using high-precision analytical tools represent an important research advancement toward clinical implementation (following much needed standardization work), perhaps using staged testing (*e.g.*, a blood Aβ test favoring sensitivity over specificity, followed by a more specific CSF- or imaging-based test in clinical practice). Here, recent advancement in high-sensitivity immunoassay, including antibody-based Single molecule array (Simoa), might prove indispensable, allowing for scaling up of clinical diagnostic, without the need for advanced mass spectrometry setups and highly trained mass spectrometry personnel (Janelidze et al., 2016). Likewise, recent developments of immuno-infrared sensor to measure Aβ (and tau) secondary structure distribution both in plasma and CSF, hold a promise of not only a sensitive and accessible measure platform, but also further diagnostic precision by expanding the Aβ (and AD) biomarker scheme to Aβ assemblies (although without their structural characterization) (Nabers et al., 2016, 2019).

12 | PRECISE CHARACTERIZATION OF Aβ ASSEMBLIES IS UNIQUELY POSSIBLE WITH MS—BUT ARE WE THERE YET?

Detection of Aβ proteoforms in brain tissue, CSF, and most recently also in plasma with the help of MS appears to have become routine. MS also holds a unique promise for precise characterization of larger Aβ assemblies, including all of the backbone sequence, bonds, and even conformation. However, are the available MS approaches sufficient to target the long-standing dogma of oligomers and, in that case, what are they?

Analyzing and producing hard evidence of the presence of dimers and oligomers of Aβ appears to be rather difficult. Already in 1985, Masters et al. detected species that very likely were dimeric Aβ using LC and SDS-PAGE (Masters et al., 1985). Few years later Roher et al. applied an alternative, SEC-based approach. to separate putative dimers and trimers, from monomers (Roher, Lowenson, Clarke, Wolkow, et al., 1993). Here the presence of Aβ proteoforms in the eluted fractions was confirmed by MALDI and PDMS. However, no MS analysis of larger species was performed. In 1999, Enya et al. combined SEC and WB showing the presence of first SDS-stable putative Aβ dimers and trimers in the water insoluble (FA-soluble) fraction of extracted brain (Enya et al., 1999). By combining different anti-Aβ antibodies structural information on both the monomeric and oligomeric species was obtained. While monomeric species ending at aa 42 showed extensive N-terminal truncation, this was not observed for monomers ending at aa 40. For dimeric species, there was no qualitative difference between Aβx-42 and Aβx-40; however, antibodies directed at the N-terminal portion of Aβ produced the

limited signal, indicating either truncation or that the epitope was blocked.

A few years later, Kalback et al. highlighted the difference between human plaque-derived Aβ and transgenic mice (Swedish mutation) including the presence of dimers in human AD brain (Kalback et al., 2002). While MS was again employed to verify the presence of Aβ proteoforms in different fractions, analysis of oligomers by MS was not performed. Still, this work demonstrated that any higher order species likely differ between one another, and that these differences depend on the Aβ sequence. This notion was further supported by Mc Donald et al. (2010) when they showed the presence of dimers in both soluble and insoluble extracts of AD brains. Later, attempts also by Mc Donald et al. to use MS to analyze such dimers isolated from brain were unsuccessful (Mc Donald et al., 2015). This work demonstrated that AD brains contain Aβ assemblies of different sizes, including rather small ~7-kDa Aβ species (likely dimers), and larger species ranging from ~30 to 150 kDa, and those larger than 160 kDa. While the smallest Aβ assemblies were shown to be highly resistant to chaotropic agents, have a higher Aβx-42/Aβx-40 ratio than the monomer fraction, and have an inaccessible/immune unreactive N-terminal, no information was obtained about the primary structure of these species.

The first successful identification of Aβ assemblies (Aβ dimers) with the help of MS and stabilizing agents was achieved only recently by Vazquez de la Torre et al. (Vázquez de la Torre et al., 2018). They applied the above-mentioned photo-induced cross-linking of unmodified proteins reaction on synthetic Aβ1-40, digested the induced dimers enzymatically and analyzed the peptides by MS. The obtained data were compared with what was obtained by collecting SEC fractions of immunopurified brain tissue, followed by enzymatic digestion and MS analysis. Thus, they were able to identify Aβ species cross-linked at Tyr10 in those samples. Finally, in 2019, Brinkmalm et al. managed to identify a number of covalently cross-linked intact Aβ peptides in plaques isolated from large amounts of brain (Brinkmalm et al., 2019). Interestingly, while a prominent cross-link between Glu22 and Asp1 was established (with the help of enzymatic digestion) in a brain from a patient with dominating Aβ1-40 profile, other samples did not contain this particular cross-link. Rather, different cross-links appeared to be present in other samples, although defining the exact position in these samples was unsuccessful.

Based on these works, it is apparent that contrary to WB, MS analysis of Aβ dimers is potentially much more informative. The MS analysis of dimers appears however much more demanding than the MS analysis of monomers. Besides low yields and special requirements, such as alkaline LC-buffers, the multitude of Aβ proteoforms gives rise to very complex mass spectra that are difficult to interpret. Still, MS offers yet another unique possibility to analyze Aβ assemblies, videlicet native MS.

Native MS, which refers to the study of assemblies (primarily proteins) in order to define their structure-function relationship, aims to retain the information about the biological status of the assemblies that these possess in solution, prior to the ESI-based ionization. These approaches rely on careful control of pH and ionic strength to maintain the native folded state of the assembly of interest. When combined with ion-mobility spectrometry that allows for the study of collision cross sections of the analytes, this setup offers the possibility to study the structural properties of different Aβ assemblies, including the distribution of different oligomeric structures (Bleiholder & Bowers, 2017; Ruotolo et al., 2007). Ion mobility has been used to demonstrate a multitude of structural properties of Aβ assemblies, although until recently primarily in vitro. These include, but are not limited to, the demonstration of: conformational conversion from random assembly to beta-sheet during amyloid fibril formation (Bleiholder et al., 2011); contribution of lysine residues within the Aβ sequence on the Aβ assembly (Lys16, Lys28) and also toxicity (Lys16) (Sinha et al., 2012); role of Gly25-Ser26 dipeptide bond in organizing Aβ42 monomer structure (Roychaudhuri et al., 2014); intra-species aggregation differences based on primary amino acid sequence (Roychaudhuri et al., 2015); and formation of distinct oligomeric species in various membrane-mimicking environments (Österlund et al., 2019). Recently, it was also used to demonstrate the effect of a drug candidate on the very early assembly of the Aβ1-40 peptide (Lazzaro et al., 2019).

Finally, in the context of fAD, it was also used to demonstrate the unique properties of two recently discovered APP mutations, the A673T (Icelandic) (Jonsson et al., 2012) and A673V (Di et al., 2009). While the A673T appears protective, the A673V mutation on the other hand results in early onset AD in homozygotes, but also appears protective in heterozygotes. Zheng et al. reported that these can be attributed to the generation of unique oligomeric species (dimers, tetramers, hexamers, and dodecamers) between both the different mutations, between mixes of the mutations with the wild-type Aβ peptides, but also between different Aβ proteoforms (Aβ1-40 and Aβ1-42, respectively) (Zheng et al., 2015). Their results suggest a potential explanation for the unique protective properties of these mutations in the context of oligomers. Furthermore, they also highlight the importance of the understudied N-terminal portion of the Aβ in its assembled form (Sgourakis et al., 2007).

13 | CONCLUSIONS AND PERSPECTIVES

The role of oligomers and their toxicity in AD is considered central dogma in modern AD research. As outlined in this review, Aβ is not a single protein/peptide but rather comprises a diversity of proteoforms and higher order assemblies. Multiple controversies regarding soluble Aβ aggregates exist, and these should not be overlooked, not least from the analytical point of view.

The general problem in common approaches to analyze the Aβ assemblies, and Aβ proteoforms as such, is that size separation techniques based on physicochemical properties and/or interactions, such as PAGE and SEC, do not provide sufficient resolution and accuracy for the correct determination of any peptide identities. Antibody-based techniques, especially in combination with separation techniques, provide a great leap forward toward peptide identification but the fine details are still elusive. MS, on the other

hand, is an extremely powerful technique for the identification of peptides, but suffers from relatively harsh requirements regarding sample preparation frequently making detection difficult. In addition, MS is often considered complicated and expensive. IP-WB with sets of cleverly chosen antibody combinations together with positive and negative controls, possibly in combination with SEC, can be an efficient way to characterize the rich flora of Aβ-related species. Still, top-down (analysis of intact endogenous peptides) and/or bottom-up (analysis of proteolytically digested samples) MS needs to be utilized for the final identification of the Aβ species. Therefore, MS approaches should be considered a requirement for any studies based on diversified Aβ peptide contents (*e.g.*, homogenates).

The current hope is that the advances in techniques, such as ion mobility spectrometry, will aid in the precise characterization of Aβ. Studies of cross-linking between adjacent Aβ peptides might aid in identifying precise components of Aβ sequence, responsible for assembly formation. The analysis of other proteins (besides Aβ) proposed to be present in the Aβ assemblies might further reveal the toxicity. Here again, MS-based approaches relying for instance on laser microdissection or imaging mass spectrometry, might pave the way to discover new targets for therapeutic intervention. Stable isotope labeling approaches, such as SILK, might not only reveal the role of distinct Aβ aggregates in AD pathogenesis, but also elucidate the timeline of their formation, as demonstrated both in plasma and CSF.

Still, given the constantly growing pool of proposed alternative biomarkers (other than Aβ) for AD, one might ask whether Aβ has a future as a biomarker for AD? In short yes, the Aβ1-42/Aβ1-40 is a reliable biomarker that reflects brain amyloidosis and has a high clinical value with a concordance between CSF Aβ1-42/Aβ1-40 (or Aβx-42/Aβx-40) and amyloid PET of about 90% (Lewczuk et al., 2017; Pannee et al., 2016). However, to capture a more detailed picture only utilizing Aβ1-42/Aβ1-40 might be an oversimplification which only to a limited extent reflects the actual high complexity of the Aβ pathogenicity. Indeed, as earlier outlined, alternative Aβ proteoforms (*e.g.*, Aβ1-34) have been identified as potential biomarkers for Aβ clearance in neurodegeneration. Likewise, N-terminally pyroglutamate-modified Aβ has received a lot of attention as a potential key aspect of AD pathology (Jawhar et al., 2011). The pGlu Aβ, generated by the glutaminyl cyclase catalyzed dehydration, has not only been closely linked to AD progression (Moro et al., 2018; Schilling et al., 2008), but was also recently shown to correlate with phosphorylation of tau at Ser202/Thr205 that itself is known to be increased in AD (Neddens et al., 2020). Furthermore, newly published studies of Donanemab, an AD immunotherapy targeting specifically targeting pGlu Aβ in Aβ plaques, were reported to both clear the Aβ plaques and slow down cognitive decline (Mintun et al., 2021). While these results make pGlu Aβ an even more promising target for AD therapeutics, both pGlu Aβ and other Aβ variants need further study to validate their usefulness in research and in the clinic. Likely, other Aβ proteoforms and especially the oligomers, also have pathogenic consequences which have yet not been elucidated. The current hope of the Aβ centric AD research lies in the elucidation of the molecular characteristic of these species, a progress which in the coming years will likely be driven by the unique capabilities of MS.

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CONFLICTS OF INTEREST

HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KB has served as a consultant, on advisory boards, or on data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program.

AUTHOR CONTRIBUTIONS

WM and GB drafted the article. All authors revised and approved the article.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable since no new data were generated for this Review article.

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