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REVIEW ARTICLE

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Mass spectrometry is a multifaceted weapon to be used in the battle against Alzheimer's disease: Amyloid beta peptides and beyond

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Amyloid-β peptide (Aβ) accumulation and aggregation have been considered for many years the main cause of Alzheimer's disease (AD), and therefore have been the principal target of investigation as well as of the proposed therapeutic approaches (Grasso [2011] Mass Spectrom Rev. 30: 347-365). However, the amyloid cascade hypothesis, which considers Aβ accumulation the only causative agent of the disease, has proven to be incomplete if not wrong. In recent years, actors such as metal ions, oxidative stress, and other cofactors have been proposed as possible co-agents or, in some cases, main causative factors of AD. In this scenario, MS investigation has proven to be fundamental to design possible diagnostic strategies of this elusive disease, as well as to understand the biomolecular mechanisms involved, in the attempt to find a possible therapeutic solution. We review the current applications of MS in the search for possible Aβ biomarkers of AD to help the diagnosis of the disease. Recent examples of the important contributions that MS has given to prove or build theories on the molecular pathways involved with such terrible disease are also reviewed.

KEYWORDS
alzheimer's disease, amyloid Beta Peptides quantitation, HHE, HNE, lipidomics, metabolomics, proteomics, 4-hydroxyhexanal, 4-hydroxynonenal

1 | INTRODUCTION

Mass spectrometry (MS) has a wide and steadily increasing range of applications, and generally is applied for analytical analyses to unveil molecular mechanisms of biological processes, test the validity of new synthetic products and study reaction mechanisms, investigate the presence of specific substances in extraterrestrial space, and understand the molecular bases of diseases as well as for diagnostic purposes. In this wide variety of applications, the contribution of MS toward the understanding of a terrible ailment such as Alzheimer's disease (AD) deserves special attention. Indeed, although the number of people affected by this disease is steadily increasing,[1] diagnostic and

Abbreviations: 2,5-DHA, dihydroxy-acetophenone; AAI, amino acid isomerization; AAR, amino acid racemization; Aβ, amyloid-β peptide; AD, Alzheimer's disease; AGE, advanced glycation end products; APP, amyloid precursor protein; APOE, apolipoprotein; CCD-UPLCMS/MS, covalent chiral derivatized ultraperformance liquid chromatography tandem mass spectrometry; CNS, central nervous system; CSF, cerebrospinal fluid; DI, direct infusion; DIA, data independent acquisition; ELISA, enzyme-linked immunosorbent assay; EOAD, early onset AD; EP, elevated pressure; FDA, food and drug administration; FI-APPI, flow injection atmospheric pressure photoionization; GC, gas chromatography; HHE, 4-hydroxyhexanal; HNE, 4-hydroxy-2-nonenal; HR/AM, high resolution/accurate mass; IDMS, isotope dilution mass spectrometry; IPL, immunoprecipitation; LA-ICP-MS, laser ablation inductively coupled plasma mass spectrometry; LC, liquid chromatography; LOAD, late onset AD; MALDI-IMS, matrix-assisted laser desorption/ionization-imaging MS; MD, microdialysis; MRI, magnetic resonance imaging; MRM, multiple reaction monitoring; MS, mass spectrometry; N-Aβ1–5, N-terminal Aβ; Nano-SIMS, nano-secondary ion mass spectrometry; NMDA, N-methyl-d-aspartate; PET, positron emission tomography; PRM, parallel reaction monitoring; PS, presenilin; PTM, posttranslational modifications; PUFA, polyunsaturated fatty acids; SID, stable isotope dilution; SPE, solid phase extraction; SPECT, single photon emission computed tomography; SRM, selected reaction monitoring; SWATH, sequential windowed acquisition of all theoretical fragmentation spectra; ToF-SIMS, time-of-flight secondary ion mass spectrometry.

therapeutic strategies are both at their infancy. Whereas the former are mainly based on tests to assess memory impairment and other thinking skills, the latter consist of symptom-alleviating drugs that do not address the cause of the disease. For these reasons, in recent years there have been a large use of various MS approaches for diagnostic as well as theranostic purposes in the field of AD. The peculiarity and the main advantages of the mass spectrometric techniques are their capability to give qualitative as well as quantitative information on the several actors that seem to be involved in AD. For this reason, MS is widely used and represents an important tool of AD investigation in laboratories and hospitals all around the world.

Here, besides an update on the recent studies conducted with MS to monitor and characterize Aβ peptides,[2] we provide an overall discussion on the wider contribution that MS has given to the understanding of the molecular mechanisms involved in AD. Hopefully, the knowledge of the possibilities that MS offers to the scientists who have decided to tackle AD will prompt new findings that eventually might allow us to win the battle against this terrible disease.

1.1 | Alzheimer's disease: Early diagnosis and cure wanted

AD can be considered a modern pathology, because its discovery and diffusion in the population is relatively recent if compared with other widespread diseases such as cancer. The first AD diagnosis was assigned to Dr Alois Alzheimer in 1906, when he first described the symptoms which included memory loss, abnormal behavior, and shrinkage of the patient's brain of a woman known as "Auguste Deter." The label "Alzheimer's disease" appeared few years later (1910) in a medical book written by the psychiatrist Emil Kraepelin, one of Alzheimer's colleagues. Dr Kraepelin distinguished this new disease from the historically familiar senile dementia and based such distinction especially on the young age of the patient, as well as other unusual characteristics such as the reported severe disturbances of speech, focal signs, and severe dementia.[3] After a century after these first observations, the search for early and reliable AD biomarkers is still at its infancy. For a complete AD diagnosis, many factors such as clinical evaluation, cognitive tests, and neuroimaging must be considered for a complete diagnosis of AD. The need for an early diagnosis is mainly dictated by a better chance for the therapeutics, because early treatment and participation in clinical trials with potential disease modifiers well in advance could help to slow down the cognitive decline. Secondly, early diagnosis could be very useful to the care givers to help the family to understand and accept the situation and to enable the patient and the family to make better planned lifestyle and legal choices. Unfortunately, if diagnosis is exclusively based on cognitive tests, then it cannot easily distinguish AD from mild cognitive impairment or normal aging, and thus the need for neuroimaging arises. In this scenario, the most-common types of neuroimaging techniques used to diagnose AD are single photon emission computed tomography (SPECT), positron emission tomography (PET),[4] and magnetic resonance imaging (MRI).[5,6] Although the former two need the administration of a radioactive imaging agents into a patient, the latter, despite being noninvasive, is quite expensive and cannot detect early stages of the disease when development starts long before any noticeable decrease in hippocampal volume or deposition of plaques and tau tangles[7,8]). Therefore, the search for new and reliable possible biomarkers in AD is still an open and widely investigated field.[9] Generally, a biomarker can be a molecule of any sort (protein, lipid, metabolite, metal ion, etc.) as long as it can be accurately measured and evaluated, to indicate normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. Importantly, in order for a molecule to be a good candidate for a biomarker, three main conditions must be fulfilled: (i) sensitivity; (ii) specificity; and (iii) ease-of-use.[10] In the case of AD, although many screening tests based on experimental measurements on biological fluids have been proposed, after more than a century, diagnostic methods had not yet met a golden standard. Because of the direct contact between the brain and the cerebrospinal fluid (CSF), the latter is considered a useful biofluid for AD diagnosis, and the first biomarkers (Aβ 1-42, total tau, and phosphorylated tau) had been identified in CSF in the 1990s.[11–13] However, only the combination of these three CSF biomarkers, which yields a combined sensitivity of >95% and a specificity of >85%, is considered valid for sporadic AD[14,15] and early diagnosis is not possible. Moreover, the diagnostic use of CSF is limited because the use of lumbar puncture is invasive and varies between countries. Therefore, the search for appropriate biomarkers from other fluids such as blood or urine still continues.[16] Particularly, other specific AD biomarkers, often based on multiplexed approaches and proteomic studies[17] have been explored and proposed, take into account the multifaceted nature of this disease. For example, although blood measures of tau and neurofilament light chain[18] are currently considered as possible blood-based biomarker candidates, further work is required to understand what role they might play in the diagnosis of AD. Although plasma/serum measurements are the gold standard in clinics because of their minimally invasiveness, they also have major drawbacks such as high contamination as well as low sensitivity.

If the search for more appropriate biomarkers is still a very active field of AD research, then the therapies nowadays available to treat AD patients are very far from being the least satisfactory. Indeed, although several prescription drugs are currently approved by the U.S. food and drug administration (FDA) to treat AD patients (rivastigmine, donepezil, memantine, etc.) none stops the disease itself and rather treat the symptoms, because they mainly intervene on biomolecular pathways that are downhill from the yet unknown cause of the disease. For example, memantine seems to block the channels of N-methyl-d-aspartate (NMDA) receptors—a glutamate receptor subfamily broadly involved in brain function.[19] It is, therefore, clear that, although such treatments can provide patients as well as caregivers with a better quality of life for a longer period of time, they cannot stop, nor even slow down the deadly outcome.

1.2 | Mass spectrometric assays to quantify Aβ peptides levels

Accumulation and aggregation of Aβ peptides have been firstly proposed as the major culprit for the development of AD and, although many other factors (see below, section 2) are now largely recognized to

be also crucial for the disease, characterization as well as quantification of Aβ peptide levels in their various oligomeric forms for diagnostic purposes have been primarily and widely investigated for many years in a variety of biological fluids and with many different analytical approaches.[20] Here, we discuss only the MS analytical methods applied to find reliable AD biomarkers based on the quantification of Aβ peptides, whereas in subsection 1.3, more recent applications of MS for the characterization of the various Aβ species are discussed.

1.2.1 | CSF

The analytical quantification of Aβ species in CSF has been considered the most direct and convenient mean to assess physiological as well as pathological processes that occur in the central nervous system (CNS) that involve these peptides.[21,22] Indeed, fractions of CNS-produced Aβ diffuse into the CSF, where they occur at concentration in the range of approximately 10-15 ng/mL[23] and play physiological roles that are not yet fully understood.[24,25] However, because Aβ accumulation and dysfunction seem to be major hallmarks of AD or at least are considered directly or indirectly involved in the etiology of the disease, it has been considered plausible to assume that altered Aβ levels in CSF as well as in other biological fluids must occur during disease progression.[26,27] It is very challenging to confirm this hypothesis with experimental evidences, mainly due to the difficulties encountered to find a reproducible and reliable method to assess Aβ levels.[28] Indeed, this peptide is very tricky to handle, and many experimental factors such as storing conditions, freeze-thaw cycles, standard preparation protocol, etc. must be taken into account in order to obtain reliable quantitative data.[29,30] Based on current data, as stated above, the use of CSF Aβ 1-42 alone or in combination with total tau and phosphorylated tau protein seems to be one of the very few biochemical analysis capable to discriminate AD compared with healthy controls of the same age (see also above), with a sensitivity and a specificity of approximately 85-90%.[31–33]

Although, in principle, it is possible to profile with high resolution differential MS complex mixtures of the proteins in CSF simultaneously and provide quantitative measurements without the need to pre-specify the analyte,[34,35] the most common experimental procedure to quantitate Aβ peptides in CSF requires a sample-preparation step. The latter is performed to extract and concentrate the peptides, which are otherwise difficult to detect because of their wide dynamic range and low abundance.[36] Many methods are currently available to purify/concentrate the Aβ peptides (solid phase extraction (SPE), immunoprecipitation (IP),[37] size exclusion, ultrafiltration and liquid-liquid extraction, immunodepletion, etc.) and the choice of the most-suitable one also depends on the kind of analysis needed. For example, if quantification as well as specific modifications of Aβ peptides must be monitored (see section 1.3), then the antibody used for IP must be carefully selected. Indeed, if a covalent modification of the peptide is expected in certain amino acidic residues such as histidine, then the antibody should be responsive to a portion of the peptide not involved with that specific modification; otherwise, it might not recognize the modified peptide. As an example, Aβ peptides covalently modified at the His residues by 4-hydroxy-2-nonenal (HNE)[38] are not recognized by Aβ antibodies specific for the 1-14 portion of the peptide chain (personal observation). Therefore, it is important to know which modifications of the Aβ peptides might be expected so to choose antibodies for the IP step that do not leave behind the modified peptides of interest. It is, therefore, very advisable to carry out preliminary in vitro experiments before the actual analysis of the biologic samples. Such in vitro experiments should mimic the conditions likely to occur in vivo (oxidative/nitrosative stress, presence of reactive species, and/or small binding molecules) and are also necessary to properly set up the correct selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) transitions for the quantitative determinations.[39,40] Use of pre-existing information about known proteins of interest, Aβ in this case, is often the key to obtain sensitive and reliable quantitative determinations with liquid chromatography (LC)/SRM or MRM methods, to exploit the unique separating and targeting capabilities of LC triple quadrupole mass spectrometers.[41,42] In a triple quadrupole configuration, an SRM/MRM analysis is commonly achieved by setting the first quadrupole to the expected precursor ion *m/z* ratio, the second one as a collision chamber, and the third quadrupole to the *m/z* ratio of an abundant fragment ion that is specific for the targeted peptide. One of the most critical aspect of the SRM/MRM approach is the selection of the proteotypic or signature Aβ peptides,[43] because only a few peptides per protein are acquired or extracted in targeted experiments. Indeed, the selection of peptides that are used for targeted protein quantitation is crucial, and it is possible to operate with or without an enzymatic digestion step, depending on the requested sensitivity. Shorter peptides usually give better signal to noise ratio, but sensitivity also strongly depends on ionization efficiency.[44–46] To take the latter into account, in order to choose the most-suitable transition to quantify the Aβ peptides, some issues must be considered: (i) the length of the peptide (approximately 10-20 amino acid residues); (ii) the possibility to distinguish the peptide from other more-abundant ones or from matrix ions; (iii) the peptide unique mass (there should not be other peptides in the sample with the same *m/z* value). Moreover, in order to ensure assay specificity, at least three peptides should be monitored for each targeted protein, and post-translational modifications should also be taken into account. The choice of the internal standard is also very important for a correct quantitation, and an isotopically labeled Aβ peptide is the optimal choice because it do has the same physicochemical properties, including chromatographic coelution, ionization efficiency, and relative distribution of fragment ion intensities.[26] The isotopic Aβ peptides must be added to the untreated samples, in order to take into account sample losses and/or contaminations during the experimental steps prior the MS analysis. In some cases, although a different peptide with similar ionization efficiency can be spiked to the purified sample just before the analysis, this procedure can be useful only to compare Aβ amounts between samples, rather than provide information about absolute Aβ concentration.[47] Other MS approaches different from MRM have been used to quantify peptides,[48,49] but sensitivity as well as reliability of such approaches cannot reach the high standard requested for Aβ quantitation with a diagnostic value. Indeed, only results obtained

with MRM and/or SRM quantitation of Aβ peptides in CSF are in good accordance with the ones obtained by other experimental techniques such as enzyme-linked immunosorbent assay (ELISA).[40]

Finally, it is important to highlight that MS, different from all the other detection techniques,[50] is capable not only to quantify a specific Aβ peptide, but also to monitor at the same time all the Aβ variants and modifications present in a sample. The latter issue is very important not only because it might prove to be a very powerful way to find new biomarkers, but also as it might give an insight into some crucial biomolecular mechanisms involved with AD. In other words, assessing the "kind" of Aβ species might be more important than quantifying them; that is, "what kind" is better than "how much" the focus of section 1.3.

1.2.2 | Plasma

Plasma is a highly desirable matrix for AD biomarker analyses because of its higher protein concentrations compared with CSF. However, a recent review analyzed all studies of Aβ detection in plasma, and no significant differences between plasma or serum concentration of Aβ markers in AD and controls were found.[15] This finding is in accordance with the view that plasma Aβ levels reflect peripheral Aβ generation which, is not connected with AD. On the contrary, total tau levels in plasma have been found significantly associated with AD. Nonetheless, several attempts to discern between AD and control patients by Aβ signatures as well as other biomarkers in plasma are present in the literature, and MS-MRM is often the method of choice.[51] Plasma is usually preferred to serum, although both are derived from blood; the former is collected in the presence of an anti-coagulant, whereas the latter is the fluid that remains after coagulation and centrifugation, processes that might cause cell lysis and contamination from the blood clot.[52] However, even in the case of plasma, finding an inter-laboratory experimental MS protocol that can assess Aβ levels in a reproducible and reliable way is a very challenging task, due to a much higher presence of contaminants than in the case of CSF. Therefore, as for CSF analyses, often it is necessary to have a clear idea of the targeted protein to be monitored in advance, in order to build up an MS-MRM method and to find the transitions relative to the targeted protein by in vitro studies performed prior the sample analysis. Once the method is obtained and the best detection limit has been achieved, a bottom-up approach is applied, where plasma proteins are first denaturized with a reductant and/or an alkylating agent and digested with a protease, commonly trypsin. After a reversed-phase separation of the tryptic peptides, the previously developed MRM method is applied, and the targeted protein level can be measured. In this way, the so-called "targeted proteomics" is performed instead of the widely used "shotgun proteomics"; the latter is severely affected by the complexity of the digested proteomes found in plasma and the wide range of protein abundances, which limit the reproducibility and the sensitivity of this stochastic approach.[53] However, because even the targeted-proteomics approach suffers from interferences from the highly complex matrix, the recently introduced high resolution/accurate mass (HR/AM) analysis is a promising alternative approach to distinguish Aβ peptides from interferences. HR/AM also allowed the development of data independent acquisition (DIA),[54] an MS approach which combines the sequential isolation of a large precursor window with full product ion spectrum acquisition. The produced data can be used for post-acquisition targeted analysis, a procedure that could be very useful in the case of immediate post-mortem samples (ie, brains, see next section),[55] for which keeping data available even though they are not considered useful at the time of their acquisition could provide new opportunities arising only with an hindsight.

The idea of DIA was also further implemented applying wider isolation windows (typically 2-25 m/z) to select multiple precursor ions for simultaneous fragmentation. The latter approach is termed sequential windowed acquisition of all theoretical fragmentation spectra (SWATH) and has been applied recently, for example, to demonstrate that advanced glycation end products (AGE) induce changes in the expression of key proteins involved in AD.[56] However, it is important to highlight that the broader m/z isolation window used in SWATH causes simultaneous fragmentation of multiple precursor ions and therefore the assignment of product ions may be difficult in some cases.

1.2.3 | Brain

MS experiments for proteomic studies on human brain samples are inherently different from the described studies carried out in CSF and plasma, because of several reasons.[57] First of all, these studies must be post-mortem, and their purpose is not for diagnosis of the disease but rather to elucidate the biomolecular mechanisms involved in AD, with the aim to identify new therapeutic strategies based on the novel findings. Moreover, large variability in proteomic studies is also observed, due to the variation in post-mortem interval before autopsy. Indeed, during the time between the death of the patient and the collection of the brain, protein degradation is observed that causes alterations in the proteomic analysis. Such alterations are particularly problematic if posttranslational modifications (PTM) or protein quantitation are the main target of the investigation. In order to tackle this problem, special rapid autopsy programs, also known as "short post-mortem interval autopsy," have been developed.[58,59] In any case, the two main kinds of MS investigations on AD brain tissues are: (i) MS proteomics studies aimed to identify and/or quantify Aβ peptides[60] as well as other proteins involved with the disease[61]; (ii) MS imaging studies aimed to obtain Aβ peptides and/or fibril distribution in the various brain regions.[62–65] In the first group of studies, IP has been often applied in order to isolate the Aβ peptides and increase the sensitivity.[66] In this way, the main isoforms of Aβ in AD brains have been identified, and it was possible to discern between controls and AD patients by comparing specific areas of the brain, the hippocampus, and the cerebellum. The limit of such an investigation remains, as in the case of other biological fluids, (see above), the use of an antibody that could not recognize Aβ peptides that are modified in the same part of the amino acidic chain responsible of the interaction with the antibody. For example, modified Aβ peptides due to PTM such as amino acid racemization (AAR) and amino acid isomerization (AAI) that occur during degradation of peptides by brain peptidases might not be subject of IP, and precious information on biochemical processes that

could be of paramount importance for the chemical understanding of the pathological processes could be lost. For this reason, other experimental approaches have been applied such as covalent chiral derivatized ultraperformance liquid chromatography tandem mass spectrometry (CCD-UPLCMS/MS), which was successfully applied to simultaneously analyze the posttranslational AAR and AAI of N-terminal Aβ (N-Aβ1−5) in the human brain.[60]

The experimental method mostly used to analyze molecules directly from brain tissues without any sample pre-treatment apart from matrix deposition is matrix-assisted laser desorption/ionization-imaging MS (MALDI-IMS). This choice is dictated by several advantages over other analytical techniques: (i) it does not require homogenization of the brain prior to sample preparation; (ii) possibility to have molecular maps of the different brain areas; (iii) wide range of detectable analytes; ie, not only Aβ peptides, but also lipids, drugs, and metabolites[67]; (iv) label-free; and (v) good lateral resolution (50-200 μm).[68] Although it has been reported that MALDI-MS detection of Aβ peptides can be affected by photo-fragmentation,[46] the latter can be used in order to identify Aβ peptides without the need to perform MS/MS experiments required for confirmation and database searches.[69] Another approach proposed to improve quantitation and sensitivity is the use of a proteolytic step prior to ionization.[70] However, in this case, it is important to properly tune the incubation time used for the enzymatic digestion, because different brain areas that have different chemical composition (eg, white and gray matters have different lipids composition) can produce biased MSI representations of the true peptide distribution. In order to circumvent such a problem, a very long incubation time to reduce missed cleavage sites almost to zero are advised, because they remove morphology-induced measurement bias. The low pH used for the enzymatic digestion of the protein decreases the risk of tissue degradation by microbial activity at long incubation time.

Although MALDI is the MS method mostly used for Aβ detection, the major drawbacks with this technique are spatial resolution, which typically is limited by the spot size of the laser beam, and the use of a matrix on top of the sample, which might interfere with detection of molecules with a molecular weight in the same range as the matrix. For these reasons, other MS approaches have been also used to monitor and spatially map Aβ peptides in brains, mainly time-of-flight secondary ion mass spectrometry (ToF-SIMS), which can detect small fragments with a spatial resolution down to ~50 nm, and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and/or nano-secondary ion mass spectrometry (Nano-SIMS).[71,72] Although ToF-SIMS is especially suitable for the analysis of lipids, proteins of interest can be imaged with a specific tagging procedure. For example, liposomes can be linked to Aβ with biotinylated antibodies and NeutrAvidin™, Rockford, IL.[73] In this way, it was possible to correlate one single Aβ protein on the brain surface to the exogenous deuterated liposomes; each contains about 200 000 lipids to result in a high sensitivity detection technique for Aβ, whose levels could be confirmed with other independent complementary analyses. Moreover, parallel ToF-SIMS analysis of endogenous lipids allows one to gather a more comprehensive picture of Aβ peptides distributions relative to other molecules. Analogously, the need to simultaneously monitor different chemical species prompted another group[60] to image Aβ peptides and metal ions in Alzheimer's plaques with LA-ICP-MS. In this case, the distribution of Aβ deposits in the brain was based on measurement of Eu- and Ni-coupled antibodies, and the laser-based methodology also allowed one to detect and map simultaneously trace element and Aβ distributions. These examples clearly demonstrate that LA-ICP-MS is capable not only to detect Aβ peptides in the brain with high sensitivity, accuracy, and strong tolerance to interference, but it also allows multi-element simultaneous detection of different analytes due to the possibility to quantify biomolecules with elemental labeling strategies.[72,74]

1.3 | "What kind" is better than "how much"

Despite the several failings of all Aβ immunotherapy approaches tested so far in the search for a possible AD cure, Aβ levels still seem to be the main concern of most scientists involved in the battle against this disease.[75,76] However, many years of research in the AD field have also found unequivocally that many other molecules must play a crucial role in the disease.[77] Indeed, abnormalities in glucose metabolism, metal ion dyshomeostasis, mitochondrial, and oxidative stress dysfunctions[78] are invariant features of AD and deserve more attention if a cure for such a multifaceted disease is to be found.[79] In this perspective, even the analysis and the quantitation of Aβ peptides must take into account not just the Aβ levels but, as already mentioned above, also the Aβ modifications caused by the above-mentioned factors. In addition, the variety of the different Aβ peptides detected in all biofluids considered above must be considered for a comprehensive and informative analysis. Particularly, Aβ peptides of variable length can be detected in vivo, and one of the reasons for such variety is that the brain is the host to several proteases capable to degrade Aβ peptides at different kinetic rates as well as with variable cleavage sites.[1,80] Therefore, from the initially secreted full-length Aβ peptide, many Aβ fragments can be produced that depend on which proteases operate the cleavage of the precursor peptides, to generate the so-called cryptic peptides.[81,82] In some cases, the latter can have a very different function from the precursor peptide,[83,84] and their specific formation depends also on the environmental conditions experienced by the particular proteases that act on the peptides. Indeed, types and abundances of the cryptic peptides generated from a precursor protein (Aβ protein in our case) can be widely affected by the presence of metal ions,[85–87] small molecules,[88,89] and pH.[90] For this reason, detailed studies of the relative abundances of the Aβ peptides are of paramount importance, because they could potentially and indirectly unveil other biomolecular mechanisms altered in AD, which are not feasible to investigate. In other words, the dyshomeostasis of Aβ could be just a consequence of another altered feature among those mentioned above, which, although not directly detectable, could be the main causative agent of the disease. Taking into account that Aβ peptides have been recognized to have important physiological roles[91,92] and that is only accumulation of the peptides that becomes pathological, catabolism of Aβ rather than its production assumes a

pivotal role in the etiology of the disease and possible therapeutic strategies could therefore target Aβ degradation; for example an increasing Aβ proteolytic enzyme's activity.[93,94]

In this new perspective, it becomes crucial to consider the relative amounts of the various Aβ species in AD and control patients to see if fragment signatures could potentially be used as a diagnostic test for AD or, at least, as indicators of an altered specific pathway that is somehow implicated in the development of the disease. Although MS represents a very valuable tool for this purpose, unfortunately, so far investigations of this kind have been sparse and results of diagnostic values are still lacking.[95,96]

2 | BEYOND THE AB PEPTIDES: THE CONTRIBUTION OF MASS SPECTROMETRY TO ALZHEIMER'S DISEASE

In recent years, MS investigations in the field of AD have not been limited to the study of Aβ peptides, but have been applied to assess and verify other possible biomarkers of the disease to try to take into account all the other factors that have been demonstrated to play a crucial role in the development of AD. Therefore, in this section, the contribution of MS to the investigation of factors different from Aβ will be discussed, whereas the search of biomarkers other than Aβ with other analytical tools, which also represent an important active contribution in the field of AD,[97,98] will not be treated here.

2.1 | Proteins other than Aβ

AD, as well as in the case of other neurodegenerative diseases, is characterized by changes in abundance, turnover, post-translational modifications, and functions of other proteins different from Aβ that lead to local changes in neuronal function.[99] For example, although not properly highlighted in this review yet, protein accumulation in the form of neurofibrillary tangles of hyper-phosphorylated tau protein is also an important hallmark of the disease. Indeed, hyper-phosphorylated tau protein accumulation has been investigated with MS almost to the same level as Aβ accumulation.[31,100,101] In all cases, it is very difficult to correlate the observed specific protein dyshomeostasis with severity of the disease and/or to draw conclusions about possible biomolecular mechanisms responsible for the development of the disease. However, the unique capability to identify and, in some cases, quantify specific protein patterns with MS has great diagnostic potentiality as well as to give the possibility to indicate the road to new therapeutic targets.[102,103] For example, MS imaging of brain tissue sections as thin as 12 μm have allowed the identification of changes in the level of specific proteins and metabolites. Such alterations clearly indicate an inflammatory state and a decrease in anti-oxidant level in the triple-knockout 3 × Tg Alzheimer mouse model.[104] This kind of result provides important indications on the possible biomolecular pathways involved in AD, and indicates possible therapeutic strategies.

On the other hand, most MS proteomics studies focus on the possibility to identify changes in the proteome between AD and control patients in an effort to develop more-reliable diagnostic tests.[105] For this purpose, many of these proteomic studies use biofluids easily obtained from patients such as plasma and CSF,[106] as already discussed above for Aβ. Proteins levels other than Aβ can be monitored and quantified by the use of high-resolution instrumentation, which allows to apply advanced MS methods such as parallel reaction monitoring (PRM). The latter is related to the SRM approach, but has the advantage of acquiring full fragment spectra instead of a choice of preselected fragments; interfering signals are avoided, whereas quantitation and high sensitivity are conserved. In this way, other biochemical pathways, and, therefore, other proteins not directly correlated to Aβ accumulation, could be monitored such as synaptic function, secretory vesicle function, and in the innate immune system.[107] In some cases, rather than scrutinize proteins levels, PTM of the proteins are the main targets of the investigations, for example in the case of the so-called "phosphoproteomics" or "glycoproteomics." In these cases, the main experimental challenge is represented by the necessity to isolate and enrich only the proteins that present a specific PTM such as the phosphorylation and/or glycosylation.[108] For this purpose, metal-based affinity chromatography, phosphoamide chemistry, and antibody-based strategies, allow one to screen proteins that, in some cases, can have a very low abundance. The latter issue, together with the high protein dynamic range, and the presence of hyper-abundant proteins such as albumin, makes the search for possible biomarkers in body fluids a very challenging task. The purpose is to identify distinct proteins in the early stages of AD in order to have an early diagnosis and to make the therapeutic strategies more fruitful.

For example, to investigate possible differences in the abundances and/or kinds of glycoproteins between control and AD samples, lectin-enrichment chromatography can concentrate target proteins, which can be subsequently identified and quantitated with trypsin digestion and LC-MS/MS. In this case, Wang et al[109] identified 645 peptides and 795 proteins, whereas 15 among the latter were differentially expressed among the three different groups considered (normal, MCI, and AD individuals). Moreover, significantly large changes in the levels of transthyretin were found in the three groups, because this protein concentration was increased in MCI patients and declined below normal level in AD. Although these kind of findings must be validated by different groups before drawing general conclusions on the biomolecular mechanisms involved in AD, they could be of paramount importance to pave the way to design new drugs to address new therapeutic targets.

Another proteomic approach to identify new AD biomarkers targets low-abundance proteins present in biofluids by depletion of the high abundant ones and application of gel-free shotgun MS.[110] However, it is very important to keep in mind that the latter method is based on specialized software to analyze the generated datasets, and the experimenter should be fully aware that the various programs normally used for this purpose could produce different, and in some cases, contradictory results to highlight the need to validate the results with independent approaches before drawing any conclusions.[111]

Finally, the search for possible proteomic biomarkers with MS methods includes also the application of imaging MS to analyze proteins in brain tissues.[112] For this purpose, the main difficulty is

represented by the protein ion suppression caused by the presence of lipids and salts. The former are very abundant in tissues and, for this reason, are usually washed away with organic solvents.[113] Very recently, supercritical fluid of CO_2 wash of lipids was applied for the same purpose, and the signal intensities of many peptides were greatly increased; to reach almost 190-fold higher than the control in some cases.[114] This example demonstrates that the experimental procedure applied for the analysis of brain tissues is absolutely crucial for the correct determination of the actual proteome present in the sample, and therefore experimenters should always validate their results with independent MS approaches before drawing any significant conclusions from the relative protein abundances found.

2.2 | Metal ions

After limits of the "amyloid hypothesis" of AD were found, the "metal hypothesis" was formulated to take into account the experimental evidence that showed the presence of metal ions (mainly copper and zinc) in the amyloid plaques[115] and their involvement in many processes linked to AD.[85,94] For this reason, MS investigations have been applied to quantify metal ions in a biological specimen to find any significantly statistically differences between AD patients and controls. For example, a coupled analysis of metal ions together with Aβ peptides in immunohistochemical sections of brains of transgenic mouse models of AD has been carried out with LA-ICP-MS, to show a good correlation between trace elements and Aβ plaques.[71] LA-ICP-MS can monitor trace elements within brain tissues of patients with a spatial resolution between about 1-100 μm to quantify them.[116] The latter feature is a specific advantage of LA-ICP-MS over other MS approaches such as SIMS. The latter has a better spatial resolution (down to 50 nm) but no valid element quantification.[117] However, the major drawback of LA-ICP-MS metal ion quantitation is the need to use internal standardization to overcome sample matrix effects due to the non-uniform distribution of all elements within the analyzed tissue. For this reason, suitable calibration and appropriate reference materials must be considered to obtain the stability and accuracy needed for meaningful and useful data.[118] Among the others, isotope dilution mass spectrometry (IDMS) is one of the most useful methods to minimize matrix effects and/or analyte losses and it has been demonstrated that quantitative imaging of Fe, Cu, and Zn in mouse brain of AD is feasible if ID is combined with LA-ICP-MS.[119]

Among the metal ions that seem to play an important role in the development of AD, special attention has been given to those capable of redox cycling between different oxidation states, that could produce oxidative stress through the Fenton reaction. In this perspective, the homeostasis of iron and copper, both present in amyloid plaques, have been widely investigated in the attempt to unveil the molecular mechanisms at the base of the disease, with special attention to the labile forms of the metals as well as the speciation of the metal-Aβ complexes.[120–123] Indeed, non-complexed metal ions are considered to be the main producer of oxidative stress.[124] Therefore, it is more important to assess speciation and local distributions of metal ions rather than overall concentrations present in a particular tissue,[125] and many studies determine the labile redox active metal ions content in brains in order to correlate metal dyshomeostasis with severity of diseases. For this purpose, microdialysis (MD) sampling has been widely applied for in vivo studies, and solutions obtained from perfused tissues have been analyzed with ICP-MS under cool-plasma operating conditions for time-resolved scanning at the *m/z* values of the metal of interest. Speciation of the latter could be achieved by operating at various pH values, because extraction efficiencies of the different metal species from minicolumns used in MD varies according to the working pH. For example, Fe(II) and Fe(III) species are mainly extracted at pH 6 and 4, respectively, so that information related to the basal concentrations of rat brain extracellular Fe(II) and Fe(III) could be obtained.[126] Therefore, because ICP-MS generates information about the amount of metals in their elemental states from samples that have been introduced into plasma, speciation of the metal-ion distribution can still be achieved with appropriate sampling procedure and special extraction methods. Moreover, extraction of samples from specific areas of the brain allows one to determine metal ion concentration differences among the various parts of the brain, to provide an insight into metal distributions between control and AD patients for each brain area. Indeed, because of cessation of blood circulation after death, metals cannot be exchanged post-mortem among the various tissues so that a spatial picture of metal distributions in the brain is feasible.[127] Intriguing findings with ICP-MS show that Zn levels are elevated in many areas of AD brains, in accordance with a situation of increased Zn^{2+} sequestration in heavily affected brain regions to cause functional unavailability of this metal ion for physiological processes in other areas, regardless of any increase in total tissue-Zn. On the contrary, although Cu increase or decrease in AD brains is still a matter of debate,[128,129] Cu levels have been reported to be depleted in many areas of AD brains[130] to possibly have a significant impact on cellular energy production, as well as to increase oxidative damage in AD brain by impairing Cu-mediated antioxidant defences such as those catalyzed by superoxide dismutase (SOD).[127]

Although the examples reported above demonstrates the possibility to apply LA-ICP-MS for bio-imaging metals concentrations of various brain areas, the limits of this approach must be taken into account. Besides the matrix effects that can be partially overcome with multipoint calibrations constructed from matrix-matched standards[131] or the double isotope dilution strategy,[132] we highlight that mapping metal ions concentrations of the brain still remains a challenging task also because of sensitivity issues[133]; only results obtained with the same experimental techniques from different areas should be compared. Indeed, systematic differences in data obtained with different experimental approaches are very much expected and must be considered.[134,135]

As mentioned above, SIMS can analyze metals in AD brains; the main advantage is a better spatial resolution and a minimum amount of sample preparation.[136] However, even in this case, the disadvantages of most MS methods are still present; that is, destruction of the sample and the intrinsic difficulty to perform SIMS experiments on samples that must withstand an ultrahigh vacuum in the analysis chamber.[68] Moreover, because metal analysis of brain tissues must be conducted

post-mortem, it cannot be used as a diagnostic tool for living patients. For this reason, attempts to analyze metal concentrations in other tissues, whose sampling would be more convenient, have been performed.[137] However, more-reliable results than those reported so far must be obtained before a feasible diagnostic method for AD based on metal ions levels can be developed.

2.3 | Metabolites

Metabolomics, that is the comprehension of chemical processes through an analysis of metabolites present in biofluids, has recently attracted much attention in the AD field.[138,139] This relatively new "omic" science, together with genomics, transcriptomics, metallomics, and proteomics, expands our detailed knowledge of systems biology—a necessary requirement if we want to win the battle against AD. Indeed, because the majority of AD clinical trials that focus on the Aβ target have failed, the need for other therapeutic approaches based on different metabolic routes is more urgent than ever.[140] It is possible to consider metabolomics as the last step of the "-omics" cascade, to be added to the central dogma of molecular biology (genes>transcripts>proteins>metabolites); it represents the level of organization closest to the organism's phenotype.[141] At variance with proteomic and metallomics studies, metabolomics requires the investigation of many different components that comprise a wide variety of compound classes with very different physical and chemical properties, as well as range of concentrations.[142] In this scenario, various MS approaches (gas chromatography [GC]-MS, LC-MS,[143] direct infusion [DI] MS,[144] and imaging MS), as well as other experimental techniques, have been widely applied alone or coupled with a separation step.[145,146] Indeed, easily available biofluids such as plasma,[147,148] urine,[149] CSF, or even saliva[150] can be interrogated with MS techniques regarding their contents in metabolites in the same way as described above for metals and proteins.

However, in order to investigate the various components of the metabolome, often various types of MS analysis must be applied.[151] As an example, if CSF must be screened, then proteins must be removed with precipitation and subsequently polar and non-polar fractions must be separated before LC-MS/MS and GC-MS analysis. This experimental approach allows one to obtain a full coverage of the metabolites present in CSF, and to provide quantitative information with stable isotope-labeled standards. In addition, SPE-LC-MS/MS must be applied to determine catecholamine and steroid levels. However, although reported results show statistically significant differences in CSF between AD patients and controls, such differences are moderate and cannot be used as biomarkers for the disease[151]; phospho-tau and Aβ1-42 still remain the best candidates for this purpose. Nonetheless, more than for other omics, metabolomic results can be interpreted in terms of altered biochemical pathways linked to pathologies[152,153] or brain structural[154] and functional[155] integrity. For example, 12 metabolites correlated with memory ability to indicate specific pathways that involve oxidative stress, inflammation, and nitric oxide bioavailability, which seem to strongly correlate with superior memory abilities[152] For this purpose, stable isotope dilution (SID)-MRM-MS produce reliable quantitative results; seven calibration standards and three quality control samples were integrated in the analytical method. Another experimental expedient aimed to improve sensitivity and accuracy of MS screening of metabolites is the combination of MALDI at elevated pressure (EP-MALDI) and ESI with obitrap analyzer based mass spectrometer. Indeed, in experiments with rat brain tissue sections, high sensitivity and dynamic range at a spatial resolution of 15-20 μm have been reported for biomolecular ions separated in mass by just 10-20 mDa, in excellent agreement with histological images.[156]

As mentioned above, GC-MS investigations of metabolites have higher sensitivity, reproducibility, and resolution than in other conventional techniques, as well as a repeatable mass spectral fragmentation obtained upon electron ionization. Such reliable mass spectra fragmentation allows a straightforward identification of peaks *via* libraries.[157] However, because only volatile metabolites, in some cases chemical derivatization is requested before detection, can be analyzed, this MS approach is restricted and often must be combined with other complementary MS techniques. For example, flow injection atmospheric pressure photoionization MS (FI-APPI-MS) has been used to analyze metabolites,[158] because it can ionize polar and non-polar compounds and eliminate a time- and sample-consuming separation step. Other important advantages of APPI over other MS approaches are low matrix effects and a linear dynamic range generally higher than that for ESI[159]; both features are of fundamental importance for metabolomics analysis. A striking aspect of the latter, as already mentioned above, is the recognition that only a thorough screening of as many metabolites as possible can provide useful information on all the possible altered biomolecular pathways to go beyond the mere Aβ cascade described by the amyloid hypothesis of AD. In this perspective, in the attempt to be as comprehensive and open-minded as possible regarding all possible cofactors that cause AD, untargeted metabolomics[160–162] has been pursued alongside a targeted one[163,164] that is, metabolomics investigations without predefining which metabolites should be measured. Analysis of this kind conducted in human plasma revealed alterations in 22 biochemical pathways, in particular polyamine metabolism and L-arginine metabolism disrupted in mild cognitive impairment subjects converting to AD.[165] However, we highlight that these kinds of studies, because of the complexity of the metabolic samples, create serious difficulties in quantitative analyses, because metabolites that span up to five orders of magnitude often occur to hinder the determination of low-abundance ones while invalidating the quantitation of high abundance ones. Among the various solutions to this problem, interesting results have been obtained by applying a data-processing method based on monitoring isotopologues, to allow one to extend successfully the linear dynamic range to five orders of magnitude.[166]

Finally, it is worthwhile to mention that the metabolomics studies comprise also the investigation of pharmacokinetic in AD; that is, screening of metabolic profiles of the administered analytes of interest between normal and AD subjects.[167] Indeed, the pharmacokinetic research carried out with the metabolomics

analyses could give a useful contribution toward the designing of active and effective drugs in AD.

2.4 | Lipids

In order to understand why lipids are also widely investigated in the AD field, it is useful to consider the risk factors for developing the disease. Indeed, although late onset AD (LOAD) has a close association with age, possession of apolipoprotein (APOE) ε4 alleles, high cholesterol levels, mid-life obesity and diabetes, early onset AD (EOAD) is closely associated with genetic factors such as mutations of the amyloid precursor protein (APP) gene and the presenilin 1 (PS1) or presenilin 2 (PS2) genes.[168] It is easy to correlate many of the above-listed risk factors for LOAD and EOAD with aberrant lipids metabolism; therefore, it is not surprising that lipid investigations has recently attracted much attention from the AD scientific community.[169,170] Moreover, phospholipids are the main constituents of brain membranes, and it has been reported that the latter, together with synaptic proteins, can increase the number of dendritic spines, the essential cytological precursor of new synapses. For this reason, supplementation of phospholipid precursors with some vitamins and other cofactors has been proposed as a possible therapeutic strategy for AD.[155] In addition, metal-ion dyshomeostasis seems to contribute to the development of the disease (see above) and it has also been recently reported that it might have a detrimental effect on lipid metabolism.[171]

In this scenario, considering that, among other pathological alterations, neurodegeneration involves also the breakdown of cell membranes to result in abnormal abundances of membrane lipids, MS has been widely applied to determine lipid biomarkers that could discriminate between AD and control patients.[172] Although the contribution of MS to lipidomics is vast, the main application of MS in the AD field is the analysis of lipids extracted from biological fluids with either direct infusion or with an HPLC step in order to improve sensitivity and separation of the different lipidic components.[173] In addition, MALDI imaging of biological tissue has also been carried out in the attempt to find post-mortem biomarkers in the lipidic component and the use of a matrix such as 2-mercaptobenzothiazole, specifically conceived to enhance lipids rather than peptides, has been used.[174] The imaging approach can monitor the distribution of lipids in different areas of the brain to allow identification of metabolic pathways that might be altered in AD models.[175] Although a separation process to identify lipids versus peptides is commonly applied, it has been reported that such a time consuming step might be avoided if one considers the different isotopic properties for the two classes of compounds.[176] Alternatively, a multimodal chemical imaging can be performed with MALDI/MS with two matrices in a multi-step approach; for example, 1,5-diaminonaphthalene for dual polarity lipid imaging in the positive- and negative-ion modes and 2,5-dihydroxyacetophenone (2,5-DHA) matrix for subsequent protein imaging.[177]

In some cases, certain lipids over others can be preferentially detected if ESI and MALDI/MS are coupled with efficient extraction and enrichment of specific lipids from the brain.[178] For example, matrices such 3-aminoquinoline are very efficient for MALDI MS analysis of gangliosides in the negative-ion mode, after a pre-treatment with ethanol and ammonium formate to distinguish specific and different gangliosides patterns between AD and control brains.[179] In any case, from the few examples reported above, it is clear that appropriate choice of matrix seems to be a critical step in the analysis of brain lipids with MALDI, as widely reported in the literature.[180]

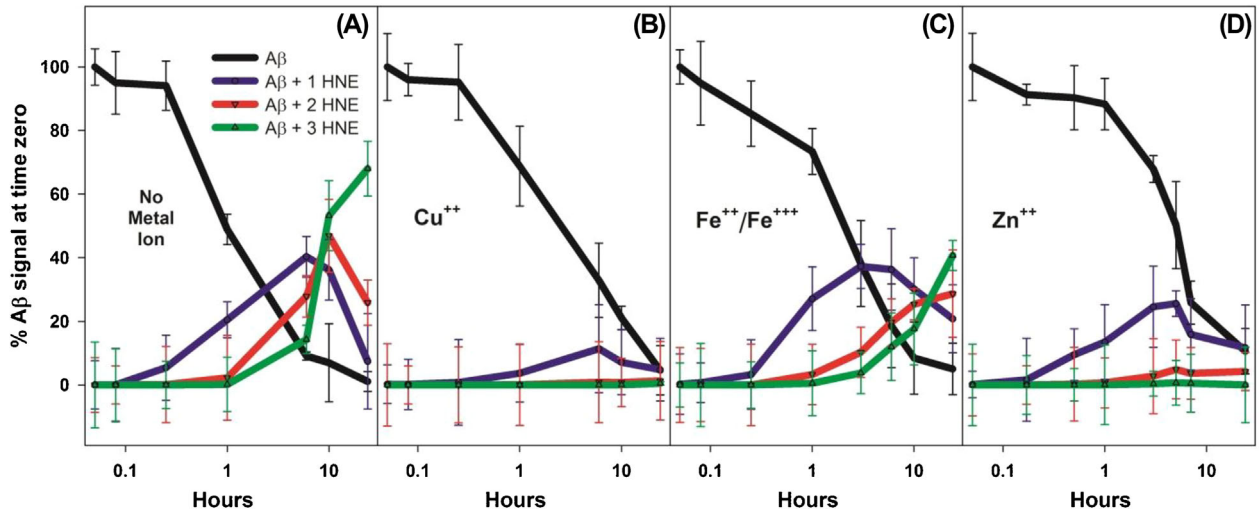

FIGURE 1 Aβ (40 μM) modification by HNE (400 μM). Aβ (black). Aβ · HNE1 (blue), Aβ · HNE2 (red), Aβ · HNE3 (green) as obtained by MRM-MS. The vertical scale in each panel represents the amount of unmodified Aβ as a percentage of the amount present at time zero (set to 0.05 h so that it could be represented on a log scale). Results were normalized with 40 μM bradykinin as an internal standard. Standard curves could not be determined for the three HNE-modified Aβ, so their signal intensities are represented as a percentage of the signal intensity for the unmodified Aβ at time zero. Consequently, signals for the four species represented do not sum to 100% after time zero. Error bars represent the standard deviations of three determinations. A, No added metal ions. B, 100 μM copper(II). C, 100 μM iron(II)/(III). D, 100 μM zinc(II)(reprinted with permission from Grasso et al[47]

Finally, an indirect monitoring of the lipidic composition of brain membranes with MS has also been largely applied to detect modifications of proteins that are produced by reactive species such as HNE or 4-hydroxyhexanal (HHE).[38,47,181] Such species are formed, respectively, by the action of oxidative stress on n-6 or n-3 poly-unsaturated fatty acids and form Michael adducts with biomolecules that contain nucleophilic moieties such as thiols and amines, including DNA and proteins. MRM-MS methods can be applied to biological samples to obtain relative amounts of HNE/HHE modified proteins. For example, in Figure 1 the kinetics of HNE addition to Aβ is reported as obtained with MRM-MS, with bradykinin as internal standard. Interestingly, protein adducts show different properties depending if they are HHE or HNE covalently modified.[182] It has been reported that whereas HNE, a product of ω-6 polyunsaturated fatty acids (PUFA) oxidation, promotes membrane association and Aβ fibril formation, HHE, the ω-3 derived analogue of HNE, does not induce such phenomenon.[183] To elucidate some of the intricate biomolecular mechanisms involved in AD, it could be crucial to determine the relative abundances of the HHE and HNE species for key proteins involved in this disease (Aβ, proteases, etc.). Indeed, further investigations in this field could provide new insights into the different roles that ω-3 and ω-6 PUFA have in the brain membranes, to explain some pragmatic observations such as the fact that ω-3 supplementation seems to be beneficial to cognitive outcome,[184] as well as vascular diseases, and to give at least some indications on dietary recommendations.[185] In this scenario, it is important to highlight that MRM/MS represents the most-suitable technique to obtain qualitative as well as quantitative information on the different HHE and HNE species, as widely reported for Aβ species.[47]

3 | CONCLUSIONS AND FUTURE PERSPECTIVES

AD is a multifaceted disease that involves several biomolecules and pathways. After many years of active research, it is now clear that it would be wrong to tackle such a disease by targeting only one of these factors, because it has been the case in the past 20 years or so for Aβ. Although the homeostasis of the latter is indeed of pivotal importance, other factors such as metal ions, lipids, proteases, and oxidative stress must be taken into serious considerations if a remedy of such incurable disease is to be found. MS, with all its various experimental approaches cited above, surely represents one of the few experimental techniques that is potentially capable to target all these factors at once, and is an irreplaceable analytical tool in the hands of researchers who study AD. However, in my opinion, a tighter collaboration between mass spectrometrists and clinicians is highly desirable because things that work in vitro often are not easily reproducible in vivo. Analogously, clinicians would draw much benefit from the interaction with experimentalists, because the latter can provide important biomolecular insights that are necessary to design a drug capable to tackle such a terrible disease.

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