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REVIEW ARTICLE

Harmonization and standardization of biofluid-based biomarker measurements for AT(N) classification in Alzheimer's disease

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Abstract

Fluid biomarkers are currently measured in cerebrospinal fluid and blood for Alzheimer's disease diagnosis and are promising targets for drug development and for patients' follow-up in clinical trials. These biomarkers have been grouped in an unbiased research framework, the amyloid (Aβ), tau, and neurodegeneration (AT[N]) biomarker system to aid patients' early diagnosis and stratification. Metrological approaches relying on mass spectrometry have been used for the development of reference materials and reference measurement procedures. Despite their excellent performances as clinical tools, fluid biomarkers often present an important between-laboratory variation. Standardization efforts were carried out on the biomarkers currently included in the AT(N) classification system, involving the collaboration of national metrology institutes, clinicians, researchers, and in vitro diagnostic providers. This article provides an overview of current activities towards standardization. These reference methods and reference materials may be used for recalibration of immunoassays and the establishment of standardized cutoff values allowing a better stratification of Alzheimer's disease patients.

KEYWORDS
Alzheimer's, AT(N), early diagnosis, fluid biomarkers, reference materials, reference measurement procedures, standardization

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Highlights

- The AT(N) biomarker system allows stratifying AD patients on the basis of biomarker profiles.
- Fluid biomarker measurements often present an important between-laboratory variation preventing the establishment of standardized cutoff values.
- Overview on the standardization initiatives involving the fluid biomarkers currently included in the AT(N) framework.

1 | INTRODUCTION

According to the World Health Organization more than 55 million people are currently living with dementia in the world, with Alzheimer's disease (AD) accounting for about 60% to 70% of the cases. In the last 20 years, the pharmaceutical industry has dedicated a substantial effort to the development of AD symptomatic and disease-modifying drugs and recently, several clinical trials[1–4] of therapeutic antibodies targeting amyloid beta (Aβ) peptides have shown positive results, both in decreasing amyloid plaque burden and in slowing cognitive decline. As therapeutic intervention is likely to be more efficient at earlier stages, accurate and early clinical diagnosis of AD will be important.

Diagnosis of AD is ensured by different approaches. Among them imaging is still widely used in clinical practice for the diagnosis and management of patients, even though it is an expensive approach, whose access is restricted to specialized centers in high-income countries.[5] Cerebrospinal fluid (CSF) biomarkers are the target of several commercially available kits for in vitro diagnostis (IVD) that allow an early diagnosis of AD and eventually the monitoring of treatment effects in clinical trials. However, the CSF-based assays present two major limitations: (1) lumbar puncture for CSF biomarkers measurement may be perceived as an invasive procedure and the possibility to have repeated sampling for clinical assessment and monitoring of therapeutics effects in clinical trials is limited[6]; for this reason, in the last few years several IVD providers have dedicated their efforts to the transfer of fluid biomarkers assays from CSF to blood and hopefully in the near future we will assist in the democratization of these tests on a large scale[7]; and (2) there is a lack of comparability among the tests, which compromises their reliability and makes it difficult to have standardized cutoffs and reference values.[8] Despite the excellent performance as clinical tools of the different fluid biomarkers, between-laboratory variation has often hindered their implementation in clinical practice, preventing the establishment of standardized cutoff values and reference ranges. Here, we provide an overview of the standardization initiatives carried out on the fluid biomarkers of AD currently included in the amyloid, tau, neurodegeneration (AT[N]) framework, relying on the development of reference methods and certified reference materials.

Different classification systems have been established in the last 20 years to aid patient stratification relying on fluid biomarkers and imaging approaches[9,10–14]: all of them aim at providing diagnostic criteria to define preclinical AD. The standardization of this definition is of utmost importance for the successful implementation of treatments and requires the assessment of the best combination of the different biomarkers for the IVD and staging of AD pathology.[15]

In 2018 a task force organized by the National Institute on Aging and Alzheimer's Association established an unbiased research framework, the AT(N) biomarker system, to enable recommendations for grouping biomarkers and stratifying AD patients on the basis of biomarker profiles.[9] This framework gives a biological definition of AD based on neuropathological changes linked to retinopathies that can be detected by imaging or biomarker levels, thus allowing the staging of the disease across its entire spectrum independently from cognition and clinical symptoms. This classification system offers a common language to researchers facilitating the standardization of research findings. In vivo CSF and imaging biomarkers have been included and divided into three categories, where "A" stands for amyloid deposition, "T" for pathologic tau, and "N" for neurodegeneration. Among the biomarkers for A (amyloid deposition), the authors included cortical amyloid positron emission tomography (PET) ligand binding and low CSF Aβ42 or low CSF Aβ42/40 ratio; as biomarkers of T (fibrillary tau), they considered elevated CSF phosphorylated tau (p-tau) and cortical tau PET ligand-binding, while the biomarkers for N (neurodegeneration or neuronal injury) were CSF total tau (t-tau), fluorodeoxyglucose PET hypometabolism, and medial temporal lobe atrophy on magnetic resonance imaging. Table 1 summarizes the AT(N) framework and the possible patients' profiles. During recent years, it has become increasingly clear that increased p-tau likely reflects an increased release of p-tau from neurons to the brain interstitial fluid and biofluids, and that the marker thus represents changed tau pathophysiology related to Aβ pathology rather than neurofibrillary tangle pathology (however, this changed tau pathophysiology may predict tangle pathology—hence, increased CSF or plasma p-tau may be a predictive marker of AD-type tangle pathology).[16]

Several studies have investigated the correlation of these cross-sectional biomarkers with the risk of developing AD and dementia. Most of them pointed out the A+ profiles as the best correlated to cognitive decline[17–19] with the A+T+N+ class being a strong predictor for AD dementia. In many other studies, some individuals were positive for tau pathology (T+) and/or neurodegeneration(N+), but negative for amyloid deposition (A−) and they could not be included in the AD group. These patients are often referred to as SNAP (suspected non-Alzheimer's disease pathophysiology) and can present dementia and amnestic mild cognitive impairment that mimic AD.[20–22] Some studies have also hypothesized Aβ-independent processes in preclinical stages of AD,[23,24] while another has investigated whether standardized cutoff values and reference ranges can help to better define SNAP by establishing if the increase in p-tau181 is correlated to amyloidopathy and thus to AD, or if it could be considered a preclinical biomarker for AD, or a biomarker for other neurodegenerative processes.[25] The diagnostic accuracy of the framework in individuals with cognitive impairment was evaluated in three cohorts, the TRIAD, the BioFINDER 1, and BioFINDER 2, showing that both A and T biomarkers in isolation had the same performance as the complete AT(N) system for distinguishing AD dementia from cognitively unimpaired individuals and patients affected by non-AD neurodegenerative diseases.[26] A recent study has evaluated the AT(N) framework as a therapeutic decision-making tool in a real-life memory clinic cohort to establish eligibility for aducanumab treatment[27]: the authors stress that the implementation of the AT(N) system into clinical practice suffers from a lack of biomarker cutoffs and they recommend the use of two different measurement modalities (fluid and imaging) to determine eligibility for the treatment. However, the establishment of biomarker cutoffs is still challenging because of the between-laboratory variability that has been observed for most of the ATN biomarkers.

The reasons for this variability can be attributed either to the use of different preanalytical and/or analytical procedures, differences in calibration, or to a lack of consistency in the manufacturing of the immunoassays, leading to batch-to-batch variability. Important efforts have been devoted to the monitoring of test results across different platforms and different laboratories: in 2009 the Alzheimer's Association quality control program for CSF and blood biomarkers was launched (https://www.gu.se/en/neuroscience-physiology/the-alzheimers-association-qc-program-for-csf-and-blood-biomarkers), together with the International Federation of Clinical Chemistry (IFCC) Working Group for CSF proteins, subsequently renamed the Working Group for Biomarkers of Neurodegenerative Diseases (https://ifcc.org/ifcc-scientific-division/sd-working-groups/csf-proteins-wg-csf/). Thanks to the standardization initiatives organized within these groups, it was possible to estimate that the between-laboratory coefficients of variation (CVs) amounted to 15% to 25%, showing a strong need for standardization of the preanalytical and analytical procedures. To this end, the establishment of preanalytical protocols for fluid biomarkers[28] and the introduction of fully automated and standardized laboratory tests[29] have opened up the way to more accurate measurements with lower between-laboratory variability.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed the literature to obtain an overview of the standardization /harmonization initiatives involving the fluid biomarkers currently included in the AT(N) framework. Metrological approaches, leading to the development of reference materials and reference measurement procedures, are illustrated.
2. **Interpretation**: Although several initiatives have been organized to harmonize fluid biomarker results for AD diagnosis, standardization is still in its infancy and collaboration among the metrological community, the clinicians, the research community, and the IVD providers is needed to ensure the establishment of harmonized cutoff values for patient stratification.
3. **Future directions**: More research is needed to reduce between-laboratory variability of AD tests and to transfer the methods from CSF to blood. It is important to establish metrological approaches leading to in-silico recalibration of AD immunoassays. This is of utmost importance to establish therapeutic interventions upon early diagnosis of AD.

However, in clinical chemistry the highest level of standardization is achieved by ensuring traceability to the International System of Units (SI) through the development of reference materials (RMs) and reference measurement procedures (RMPs). While research on AD has significantly progressed in the last few years through the discovery of new biomarkers and the development of non-invasive sensitive assays implemented on automated platforms, large-scale implementation of these biomarkers in routine clinical settings necessitates standardization. To date, most initiatives striving for standardization of fluid biomarkers are focused measurements performed in CSF, including A$\beta_{1\text{-}40}$, A$\beta_{1\text{-}42}$, t-tau, p-tau181, and neurofilament light chain (NfL), another candidate for reflecting neurodegeneration in the AT(N) system.[30] Metrology plays a key role in supporting the implementation of those biomarkers into clinics, by providing the required traceability for calibrators, frameworks for measurement uncertainty, and reference measurement systems to enable measurement standardization and establishment of relevant clinical thresholds.

To this end, the European Metrology Programme for Innovation and Research (EMPIR) has funded two projects, NeuroMET 15HLT04 (2016 to 2019),[31] and NeuroMET2 18HLT09 (2019 to 2022),[32] in the field of neurodegenerative diseases. These projects aimed at improving measurement capabilities for effective screening and diagnosis of neurodegenerative diseases (NDD) and developing a framework for early diagnosis in a multidisciplinary approach combining imaging, cognitive assessment, and biomarkers. This work led to important advances, paving the way for tight collaboration among

TABLE 1 Illustration of the different AT(N) classes and patient profiles

AT(N) Class	Fluid Biomarker	Imaging
A	CSF or blood Aβ	Amyloid PET
T	CSF or blood p-tau	Tau PET
N	CSF or blood t-tau, NfL	Metabolic PET, MRI

AT(N) profiles				
Profile	**A**	**T**	**N**	**Classification**
A− T− (N)−	−	−	−	No AD
A+ T− (N)−	+	−	−	Possible AD
A+ T+ (N)−	+	+	−	AD
A+ T+ (N)+	+	+	+	AD
A+ T− (N)+	+	−	+	Early AD and/or amyloid-positive individual with neurodegeneration
A− T+ (N) −	−	+	−	Unusual pattern of unclear clinical relevance
A− T− (N)+	−	−	+	Non-AD neurodegenerative disease
A− T+ (N)+	−	+	+	Non-AD neurodegenerative disease

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; AT(N), amyloid, tau, and neurodegeneration; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; NfL, neurofilament light chain; PET, positron emission tomography.

metrology laboratories, clinicians, assay manufacturers, and various key institutions including the IFCC Working Group for Biomarkers of Neurodegenerative Diseases, the Michael J. Fox Foundation, EUFIND, and the Joint Committee for Traceability in Laboratory Medicine (JCTLM). An important part of these projects was devoted to the development of reference methods and reference materials for fluid biomarkers.

2 | METROLOGICAL APPROACHES TO ENSURE MEASUREMENT TRACEABILITY

Immunoassays for biological fluid biomarkers, if properly standardized, should allow highly accurate measurements. The objective of harmonization and standardization initiatives is to provide equivalent results among different measurement procedures, thus ensuring the establishment of common reference intervals and cutoff values, facilitating medical decisions and patient care services. The purpose of this section is to explain how to achieve traceability of the results from routine measurement procedures to higher order RMs, and what are the characteristics of such RMs in terms of purity and uncertainty and how these materials are value-assigned. The ISO standard 17511:2020 defines how to establish metrological traceability of values assigned to calibrators, trueness control materials, and human samples for quantities measured by IVD medical devices and describes six categories of metrological traceable calibration hierarchies, of which the most desirable is the one providing traceability to a primary RM and/or RMP.[33] In clinical chemistry, the highest level of standardization for end user measurement procedures is achieved through the development of RMs and/or RMPs in order to establish a calibration hierarchy to the SI. When SI-traceable RMs and RMPs are not available, it is still possible to obtain equivalent, non-SI-traceable values among different routine measurement procedures through a process called harmonization. As described in ISO 21151, each IVD manufacturer develops a method-specific correction algorithm to achieve equivalent results for clinical samples. Metrological traceability to SI units offers the advantage of established references that are stable over time and space and can be more easily reproduced.[34] Figure 1 illustrates a traceability chain for clinical sample results.

For each measurement procedure, it is important to define the quantity intended to be measured, referred to as the measurand. The AT(N) framework includes protein biomarkers in CSF and blood. Due to the rapid advancements in biological mass spectrometry (MS), this technique is often used for detailed molecular characterization and quantification of the analytes by MS, and development of RMPs and certified RMs (CRMs).

Protein measurands are commonly heterogeneous molecules, whose measurement is often complicated by the co-existence of multiple proteoforms in biological fluids, due to processing events, post-translational modifications (PTMs), and eventually alternative splicing events, plus the presence of higher order structures leading to different structural conformers.

In order to ensure traceability to the SI of the candidate RMP, it is necessary to develop calibration materials that can be constituted by peptide or protein material. The use of unlabeled synthetic peptide standards as primary calibrators can be coupled to isotope-labeled peptide internal standards, isotopologues to the target proteotypic peptides, to obtain calibration blends which will be analyzed by liquid chromatography-isotope dilution (LC-ID) MS. The use of intact protein primary calibrators is also possible and can be associated to stable isotope-labeled protein internal standards to compensate for sample preparation, sample loss, and digestion incompleteness.

Candidate primary calibrators generally consist of pure peptide/protein materials of well-characterized purity, whose mass

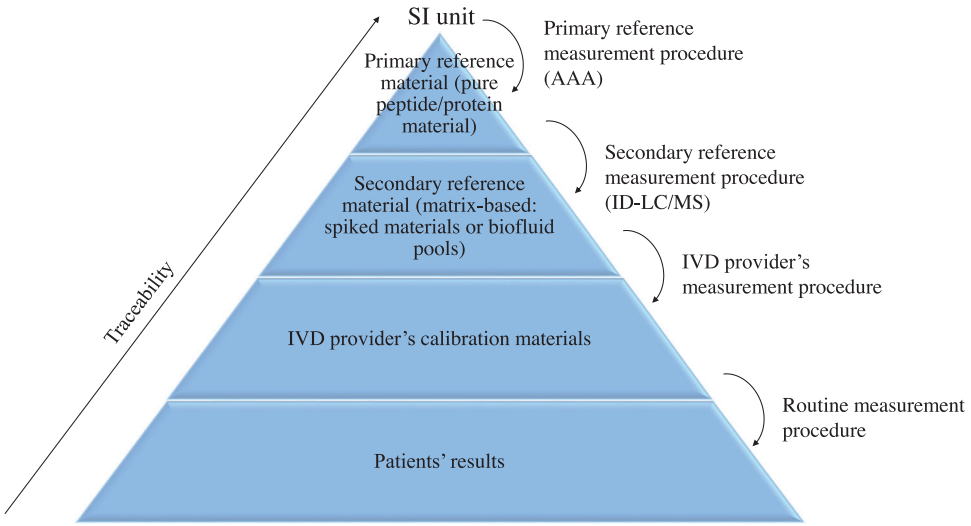

FIGURE 1 Traceability chain for clinical sample results. AAA, amino acid analysis; ID-LC/MS, isotope dilution liquid chromatography/mass spectrometry; IVD, in vitro diagnostics; SI, International System of Units

fraction can be determined by different higher order approaches, the most common of which are mass balance and peptide impurity corrected amino acids analysis (PICAA). The absolute quantification of the peptide/protein material by PICAA requires a lower amount of material and can be performed according to protocols including a complete hydrolysis of the material followed by the MS-based quantification of stable amino acids. Figure 2 describes the characterization of a candidate primary calibrator by using the PICAA approach. SI-traceability can be ensured through the use of amino acids that are certified reference materials. The mass fraction value for the peptide/protein material is obtained by the values of mass fraction of the individual amino acids and needs to be corrected for the presence of the eventual amino acid-containing impurities. As all amino acid-containing impurities need to be identified and accurately quantified, the purity assessment of the material is an important step for the value assignment of these materials.[35]

Mass spectrometry (MS)-based protein reference measurement procedures are generally based on the bottom-up approach, consisting of the digestion of the sample by a proteolytic enzyme and the subsequent analysis by MS of specific proteotypic peptides. This peptide-centric approach allows the absolute quantification of protein biomarkers in biological fluids by ID coupled to targeted MS approaches, that is, selected reaction monitoring, also known as multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM).[36,37] MS can be coupled to antibodies-free sample preparation workflows but in case of biomarkers with a very low concentration in the biological fluid, the use of immunoprecipitation can be envisaged. This is the case when transferring the methods for AD biomarker from CSF to blood. Figure 3 describes a possible schema for the development of an RMP for a protein biomarker.

The analytical performance of a candidate RMP can be evaluated by method validation on matrix-matched materials. The criteria for method validation can include linearity, selectivity, trueness, precision, autosampler stability, limits of quantification, and carryover by using fit-for-purpose quality control samples.

An important step in the development of an RMP is the estimation of the standard uncertainty which is a measurement of the probability or confidence level on the results. The producers of CRMs are required to include contributions from the different elements to the total uncertainty budget in compliance to the ISO 17034 standard and to the Guide to the Expression of the Uncertainty in Measurment.[38] In general, all the uncertainty components associated with the measurement are combined by taking into account the uncertainties associated to the gravimetric preparation of the samples and the uncertainty associated to the calibration. This includes uncertainties related to the calibration regression model and uncertainties associated with the gravimetric preparation and value assignment of the calibration blends by amino acid analysis (AAA) or other methods. It is possible to include a precision component that reflects the variance among the independent replicates.

In this article we will analyze the progress achieved on standardization/harmonization of the different fluid biomarkers currently included in the AT(N) framework.

3 | A: Ab

The A$\beta_{1\text{-}42}$ peptide is one of the most clinically relevant biomarkers for AD: the decrease in the concentration of this peptide in CSF is one of the major hallmarks of AD allowing an early diagnosis of the disease many years before the onset of the symptoms. The CSF Aβ42/Aβ40 ratio reveals even better performance in predicting amyloid positivity and interest in it is progressively increasing.[39]

Two reference measurement procedures for A$\beta_{1\text{-}42}$ (C12RMP1 and C11RMP9) and one for A$\beta_{1\text{-}40}$ (C16RMP2R) are currently listed in the JCTLM database, which established and maintains a list of higher order

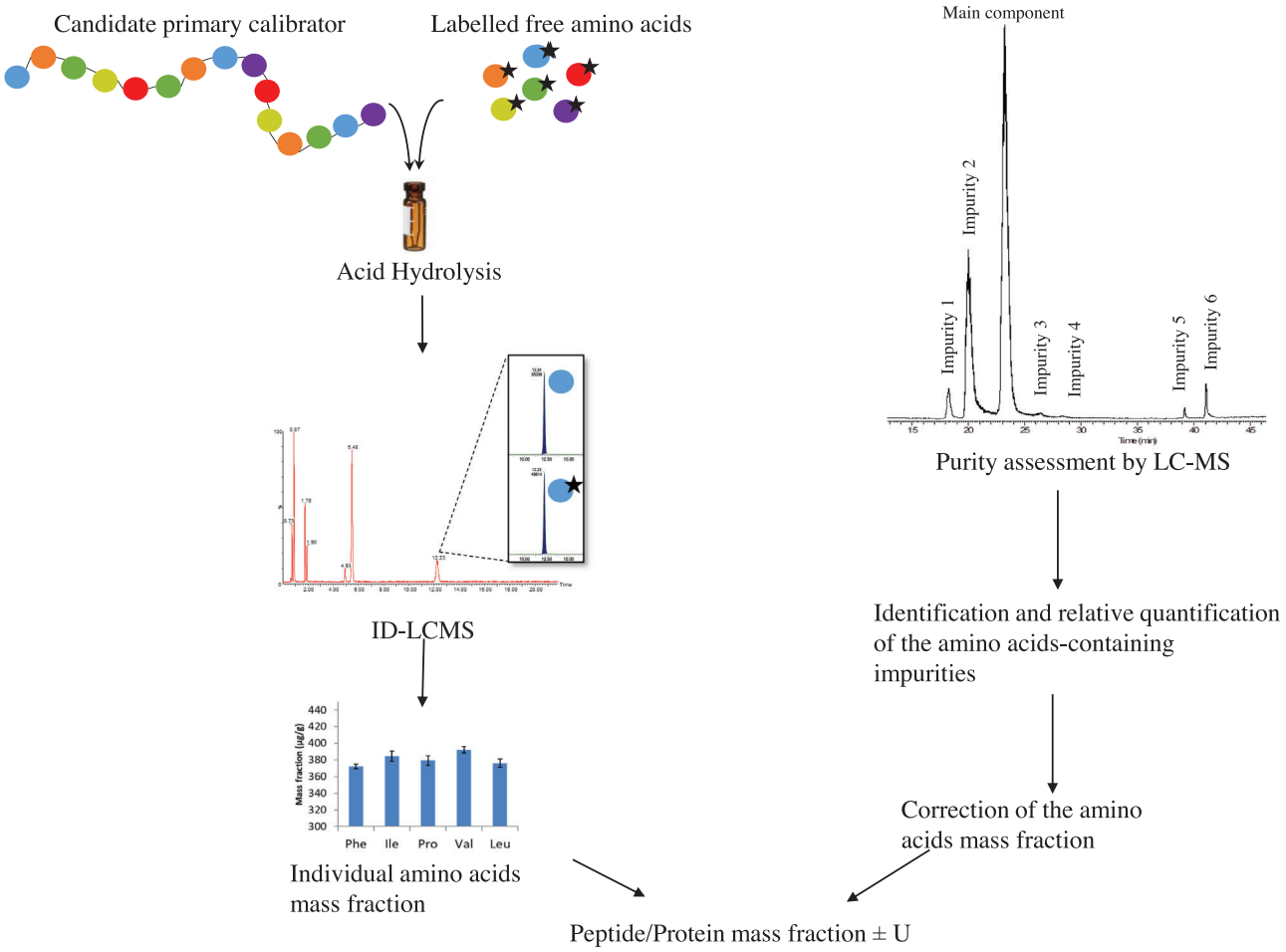

FIGURE 2 Characterization of a candidate primary calibrator by using the peptide impurity corrected amino acids analysis (PICAA) approach. The peptide/protein material is hydrolyzed after the spiking of isotope labelled-free amino acids and analyzed by isotope dilution liquid chromatography mass spectrometry (ID-LCMS). The mass fraction of the peptide/protein material is determined by the mass fractions of the individual amino acids and can be corrected for the presence of amino acid-containing impurities. The value obtained for the mass fraction is accompanied by an uncertainty, indicating the confidence level on the results

reference methods (ISO 15193 compliant) and RMs (ISO 15194 compliant), and also reference measurement service providers (ISO 15195 and ISO 17025 compliant).

The C12RMP1 method for Aβ42 was developed by Korecka et al.[40] (University of Pennsylvania) and was based on a calibration approach relying on an unlabeled Aβ42 calibrator spiked in a matrix of artificial CSF plus bovine serum albumin, together with a [^{15}N]Aβ42 internal standard. The applicability of this matrix was assessed by the method of standard additions. Sample preparation included a treatment with guanidine hydrochloride (GuHCl) followed by μSPE on Oasis MCX cartridges. The 2D ultra performance LC (UPLC) separation at 60°C, including a trapping column for sample desalting and a C-18 eluting column, was followed by MS detection in MRM mode on an API 5000 triple quadrupole mass spectrometer (AB Sciex). The range of applicability of the method was between 100 and 3000 pg/mL. The lower limit of detection (LLOD) was estimated at 50 pg/mL and the expected uncertainty was between 14.3 pg/mL and 355.2 pg/mL.

The C11RMP9 method for Aβ42 was developed on behalf of the IFCC Scientific Division Working Group on CSF proteins.[41] The method relied on a surrogate analyte approach, consisting of a calibration strategy involving two different isotopically labeled standards, the first one ([^{13}C]Aβ42) acting as a labeled internal standard in calibration blends and samples, and the second one ([^{15}N]Aβ42) as a calibrator to generate the calibration curve. The reason for this is that it was not possible to develop artificial matrices as Aβ42 behaves differently in other protein-containing buffers and it was necessary to use CSF to produce calibration materials. However, Aβ42-free CSF is not available. The method was developed on a quadrupole-Orbitrap hybrid mass spectrometer (Q Exactive) with the possibility of transfer on triple quadrupole instruments and validated according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use guideline Q2(R1)[42] and ISO 15193.[43] The [^{15}N]Aβ42 material was value-assigned by comparing its MS signal to the signal obtained from a native Aβ42 with known concentration determined by AAA. The concentration of the endogenous Aβ42 in unknown CSF was then calculated by comparing the Aβ42/[^{13}C]Aβ42 ratio of CSF samples with the calibration curve ([^{15}N]Aβ42/[^{13}C]Aβ42). The LLOD of the method was determined at

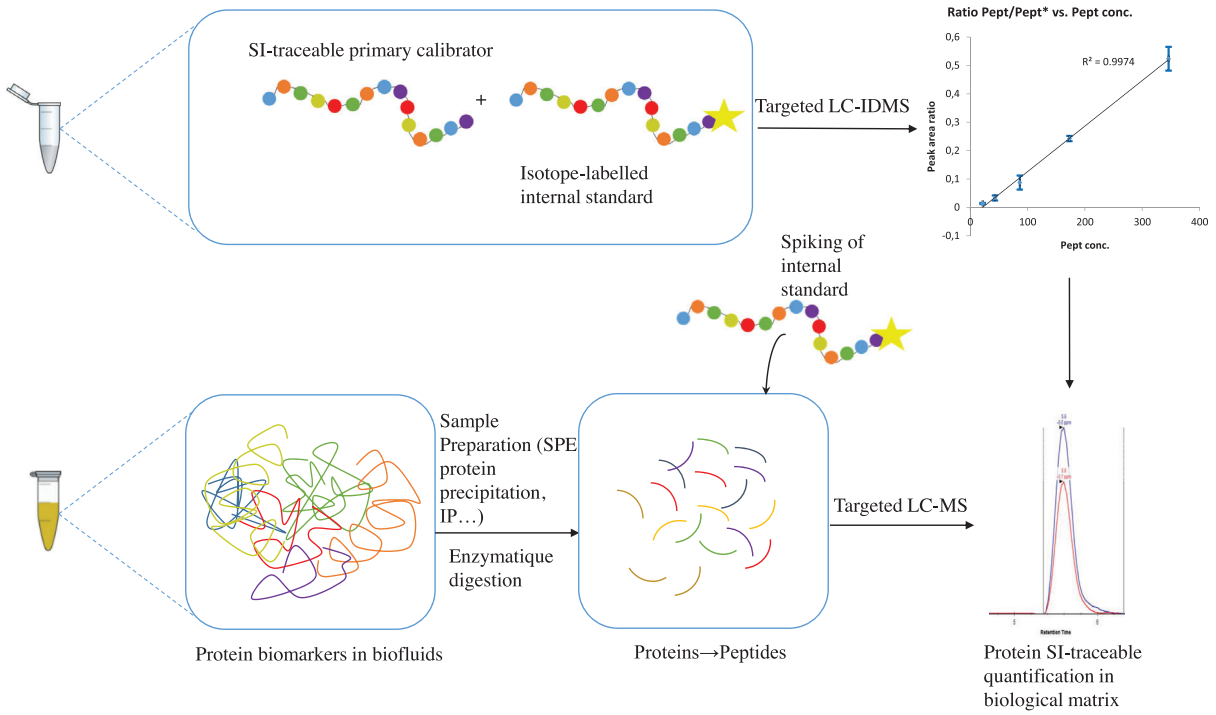

FIGURE 3 Possible scheme for the development of a reference measurement procedure for a protein biomarker by using a bottom-up proteomics approach consisting in the digestion of the proteins by a proteolytic enzyme. The use of well-characterized primary calibrators ensures traceability to the International System of Units (SI) and the spiking of isotope-labelled internal standards allows liquid chromatography isotope dilution mass spectrometry (LC-IDMS). Calibration blends are prepared by mixing different amounts of primary calibrators and internal standards to obtain a calibration curve that will allow the quantification of the protein biomarker in the biological fluid

150 pg/mL and the expanded uncertainty at the concentration 700 pg/mL was estimated at 15.7%.

The method C16RMP2R was developed by the University of Pennsylvania and targets the quantification of Aβ40 in human CSF by UPLC-tandem MS in a range of 200 to 20,000 pg/mL with an uncertainty ranging from 17.4 to 1084.6 pg/mL. The method was developed in the context of a larger study including the three peptides $A\beta_{1\text{-}38}$, $A\beta_{1\text{-}40}$, and $A\beta_{1\text{-}42}$, and is applicable to standardize the determination of Aβ42/Aβ40 ratio for improved detection of amyloid pathology.[44] The method was applied to the ADNIGO/2 patient cohort revealing an improved concordance of the CSF Aβ42/Aβ40 ratio with florbetapir-PET. Efforts are still ongoing to develop an SI-traceable primary CRM for Aβ40.

Two round robin studies were organized to determine the interlaboratory variation between different LC-MS/MS measurement procedures. A first inter-comparison[45] was carried out among the University of Gothenburg (UGOT), the University of Pennsylvania, Waters Corporation, and PPD Laboratories. Twelve human CSF pools were provided to each participant to be analyzed as for the quantification of Aβ42 through their LC-MS/MS in-house validated procedure and by using their own calibration procedure. MS results (obtained by using triple quadrupole equipment) were also compared to the results obtained by enzyme-linked immunosorbent assay (ELISA) (INNOTEST β-amyloid [1-42] from Fujirebio) and presented a quite high correlation with the ELISA results ($r^2 > 0.85$). The interlaboratory correlation was also very good ($r^2 > 0.98$), the average intralaboratory CV was 4.7%, and the average interlaboratory CV was 12.2%. The interlaboratory variation can be attributed to the use of different calibration materials/procedures. A second round robin study was thus organized to investigate the impact of using a common calibrator on the final MS results. The Joint Research Centre (JRC) produced an SI-traceable calibrator for Aβ42 by assigning the mass fraction by AAA. The calibrator was provided to the four participant laboratories (UGOT, University of Pennsylvania, Waters Corporation, and Roche Diagnostics GmbH) together with 20 CSF single donations.[46] The correlation of the data among the different laboratories was good and the interlaboratory CV was 9%.

Two commutability studies were organized to evaluate the suitability for candidate matrix materials for the production of secondary CRMs. The first study evaluated the commutability of 16 candidate matrix-based CRMs including CSF pools and artificial matrices such as diluted serum and phosphate buffered saline spiked with synthetic Aβ42.[47] These samples were analyzed together with 48 CSF single donations by using eight immunoassays (Elecsys, EUROIMMUN, IBL, INNO-BIA AlzBio3, INNOTEST, MSD, Simoa, and Saladax) and an MS-based method. The study concluded on the commutability of the neat CSF and they assessed the good correlation of the results across the different methods. A second study investigated the commutability of CSF pools for Aβ42, Aβ40, and t-tau and p-tau by using different immunoassays (EUROIMMUN, Fujirebio, IBL, Meso Scale Diagnostics and Roche).[48] For Aβ40 and Aβ42 it was also possible to use an MS-based procedure. The study confirmed the commutability of the neat

CSFs pools for Aβ42, whereas the results for the other biomarkers showed the necessity to produce CRMs with concentrations fitting the individual biomarker concentration in CSF.

Based on the results from the commutability studies, three CSF pools with Aβ42 concentrations ranging from the low to the high ends of the clinical range were developed by the JRC and analyzed by Roche Diagnostic with the Elecsys immunoassay and by ADx Neurosciences with the EUROIMMUN assays to evaluate stability and homogeneity. The between-unit uncertainty (u_{bb}) was below 1.5% for the three pools that are now available as CRMs (ERM-DA480/IFCC, ERM-DA481/IFCC, ERM-DA482/IFCC), and referenced in the JCTLM database.

The impact of the use of Aβ42 CRMs in recalibrating three commercial immunoassays (Lumipulse G series from Fujirebio, Elecsys from Roche, and EUROIMMUN Chemiluminescence [ChLIA] RA 10 analyzer from EUROIMMUN) was investigated.[49] Before recalibration the bias at the median concentration of 700 pg/mL across platforms was between −12% and 49%. Following recalibration it was possible to reduce the bias, obtaining values between −2.06% and 1.16%.

The use of blood-based assays to measure Aβ deposition has been recently introduced and constitutes an attractive alternative to CSF-based measurements, avoiding invasive sample collection. A standardized operating procedure for plasma handling has recently been published,[50] and a round robin on plasma Aβ methods was organized among 10 participant centers within the Global Alzheimer's Association[51] by using seven immunological assays and four MS methods, showing a weak correlation for Aβ42 and a stronger one for Aβ40. Better results will be probably obtained in the near future with the optimization of the measurements in plasma, the application of standardized operating procedures, and the eventual use of common calibration materials.

4 | T: P-TAU

Tau hyper-phosphorylation is one of the main hallmarks of AD. The detection and quantification of this modification occurring on serine and threonine amino acids provides higher accuracy to discriminate AD from other forms of dementia and to stratify patients at an early stage. Commercially available immunoassays are able to target specific phosphorylated epitopes: CSF p-tau181 is a well-established biomarker, routinely measured in clinical practice, whereas p-tau217 and p-tau231 are emerging tools for early diagnosis and patient stratification, allowing a preclinical assessment of the diseases in cognitively unimpaired patients. The standardization of these novel biomarkers can contribute to their uptake in clinical practice by the establishment of standardized reference ranges and clinical cutoffs.

According to the AT(N) classification, high levels of p-tau on threonine 181 reflect or predict neurofibrillary tangle pathology. Tau contains about 80 putative phosphorylation sites and extensive identification of these sites in CSF and in brain have been performed with the confirmation of 29 phosphorylation sites from brain lysates and 12 phosphopeptides in the CSF.[52] A direct link between Aβ and p-tau species has been explored by Horie et al.,[53] starting from the evidence that hyperphosphorylation at T217 increases with the amyloid deposition measured by PET, more than 20 years before symptoms and before tau aggregation. The degree of phosphorylation in soluble brain p-tau was measured and found linearly associated with Aβ, concerning pT111, pT153, pT205, pS208 and pT217, with pT217 exhibiting the best association. Insoluble tau presented a higher phosphorylation than soluble tau. Investigations on two different cohort underlined the specificity of p-tau181 and p-tau217 isoforms as AD biomarkers in preclinical and advanced AD by using a targeted LC-MS method coupled to tau immunoprecipitation. The results on both cohorts revealed that p-tau217 is able to differentiate between AD patients and other neurodegenerative diseases.[52]

During the NeuroMET2 18HLT09 project, a study was carried out to evaluate the feasibility for the development of an SI-traceable quantification of the particular proteoform of tau bearing a phosphorylation on threonine 181 (p-tau181). First, a primary calibrator consisting of a synthetic peptide was sourced and its purity was thoroughly characterized prior to certifying concentration in calibration solutions by amino acid analysis. These calibration solutions were then used to calibrate the candidate RMP for p-tau181 quantification in CSF with measurement results traceable to the SI units. T-tau primary calibrator was also used to allow the multiplexing quantification of t-tau and p-tau181. Important work was done to optimize sample preparation, chromatographic conditions, and mass spectrometry conditions in order to reach an appropriate limit of quantification with sufficiently low measurement uncertainty. Method development and validation were conducted using three pools of frozen CSF that covered low, medium, and high p-tau181 concentrations. These pools were successfully measured by the candidate reference method, demonstrating its suitability to the certification of furtherly developed CRMs (manuscript in preparation). A major challenge in the development of primary calibrators for p-tau is the presence of multiple phosphorylation sites that make the development of a unique protein reference material very difficult.

The need for multiplexing approaches to target simultaneously several phosphorylation sites is fulfilled by mass spectrometry, which can be seen not only as a technique for the development of reference measurement procedures but also as an approach for identification of other clinically relevant phosphoforms by using synthetic modified peptides as calibration materials.

Recently, a multiplexing antibody-free LC-MS method, targeting a panel of phospho-epitopes (pT181, pS199, pS202, pT205, pT217, pT231, and pS396) in CSF, has been developed by using isotope-labelled phosphorylated internal standards and a PRM approach.[54] Results were compared to the p-tau immunoassay results obtained on the Simoa platform. The method was also applied to a patient cohort comprising the entire Alzheimer's disease spectrum, confirming the importance of p-tau217 and p-tau231 as early biomarkers of AD. The results achieved in this study can constitute a preliminary step in the development of an RMP targeting the clinically relevant phosphorylation sites of tau.

The finding that blood communicates with brain across the blood-brain barrier has recently paved the way for the development of blood-based assays for tau protein. However, the current trend of performing NDD biomarker measurements in blood warrants the use of ultra-sensitive methods for the detection of sub-stoichiometric modifications of blood biomarkers which are present at very low concentrations. Recent studies in preclinical AD patients have shown that blood measures on different biomarkers (p-tau181, p-tau217, p-tau231, Aβ42/40, NfL, and glial fibrillary acidic protein) indicate p-tau217 and p-tau231 as the earliest changes in preclinical patients.[55] Another study targeting Aβ42/40, p-tau217, and NfL in unimpaired elderly populations found that the combination of these biomarkers can identify individuals at risk of developing AD dementia.[56] A comparison among six different assays targeting p-tau isoforms 181, 217, and 231 on the Simoa platform showed good clinical performance in terms of reproducibility. However, the authors underline the need for improving comparability of results provided by the different assays, using commutable reference materials for the harmonization of plasma p-tau measurements.[57] Another study recently compared 10 Simoa-based assays with an MS-based method to measure p-tau217, showing that this method is the best performing in the identification of mild cognitive impairment patients and patients progressing to dementia.[58]

5 | N: T-TAU

T-tau in CSF is routinely measured as a diagnostic biomarker of AD and is employed for patient stratification. Its concentration is correlated with AD progression, although it is now considered a less specific biomarker than p-tau, helping to better define the extent of neurodegeneration and of cognitive decline. In the AT(N) biomarker framework it is included as a marker of neurodegeneration/neuronal injury (N). The importance of establishing cut-off values for t-tau in the AT(N) diagnostic criteria has been underlined in several works, all reporting on the heterogeneity of CSF t-tau threshold values, and on the need for harmonization of routine measurement procedures.[59]

Tau is a highly heterogeneous protein, characterized by the presence of multiple proteoforms in biological fluids, due to alternative splicing, processing, and PTMs. These sources of variability, together with the coexistence of different conformers, can contribute to the variability among different measurement procedures, leading to variations in the selectivity of immunoassays.[46,48,60,61]

To date, several LC-MS methods targeting t-tau in CSF have been published,[62–67] all relying either on triple quadrupole equipment or high-resolution MS systems, and different sample preparation procedures. The major difficulty in developing MS-based quantification methods for tau is to select proteotypic peptides to be used as quantifiers that are not subjected to PTMs and that represent most of the tau proteoforms present in CSF. To this purpose, there is a consensus on the use of the peptide 156-163 (GAAPPGQK) for t-tau quantification. A candidate reference measurement procedure[68] targeting this peptide in CSF was recently validated by exploiting the potential of ID LC-MS/MS and the use of a fully characterized SI-traceable protein primary calibrator. The mass fraction of this calibrator was determined by AAA and purity was assessed by LC-MS at high resolution. The RMP was validated with a LLOQ of 0.5 ng/g, covering t-tau concentration in healthy donors' CSF and was applied to three CSF pools corresponding to three different concentration ranges (low, medium, high). The relative expanded uncertainty on t-tau mass fraction was under 10% for the three CSF pools, with the uncertainty on the primary calibrator concentration being the main source on the uncertainty budget (70%), followed by the uncertainty associated with the gravimetric preparation of calibration blends (27%). The three pools of CSF were also analyzed by the INNOTEST hTAU-Ag immunoassay, exhibiting a good correlation between this test and the RMP, but higher values were assigned to the t-tau concentration when using the LC-MS method. This discrepancy was already reported in other works[65,66] and needs to be investigated.

A round robin study was organized within the IFCC WG on CSF proteins between the University of Gothenburg (UGOT), the Laboratoire national de metrologie et d'essais (LNE), and the University of Pennsylvania (UPENN), with the aim of evaluating whether the different calibration laboratories provide equivalent results in measuring t-tau concentration in unknown CSF samples. All the participants used an LC-MS method and the bottom-up approach to measure the peptide 156-163. LNE and UPENN used recombinant protein calibration materials (LNE's primary calibrator was SI-traceably quantified by AAA), whereas UGOT used a particular peptide material, the wing-peptide, having the 156-163 sequence surrounded by two wing sequences at the N- and C-term (this material was SI-traceable quantified by AAA). As a whole, we can affirm that all the values provided by the participants to the study are very far from the data obtained by immunoassay, implying that recalibrating immunoassays against a mass spectrometry-based reference method would cause a major shift in results provided by immunoassays. The ID LC-MS/MS methods from LNE and UPENN gave higher results than immunoassays for all CSF samples. Results from UGOT were lower than the nominal concentration obtained by Lumipulse. This could be due to the use of the wing-peptide material. Regardless, even if there was no agreement among the data from the individual laboratories, results were well correlated, with the square of the Pearson correlation coefficient as $r^2 > 0.978$. It can be hypothesized that standardizing calibration could allow improving the consistency of results: the use of an SI-traceable calibration material by all the laboratories could allow obtaining better agreement between results provided by the different MS-based methods.

The production of commutable CRMs has also been addressed by a commutability study organized by LNE during the NeuroMET and NeuroMET2 projects for the assessment of 13 matrix-based CRMs involving eight immunoassays from five IVD providers and following the IFCC's latest recommendations. The materials found to be commutable will be value assigned by using the reference measurement procedure developed. The involvement of the assay manufacturers will pave the road toward the establishment of an external quality assurance scheme to assess the accuracy and reproducibility of common methods before and after standardization.

Study cohorts have concluded that blood t-tau is not diagnostically useful as it shows low correlation with CSF tau[69] and no standardization initiatives in blood have been organized.

6 | N: NFL

NfL, a neuroaxonal intermediate filament protein, is a major component of large myelinated axons[70] and important for axonal stability and growth.[71] Upon neuroaxonal damage, NfL is released into the CSF and subsequently into the bloodstream, where it can be detected using ultrasensitive immunoassays. It is also included in the AT(N) classification as a biomarker of neurodegeneration in CSF and blood.[30,72] A recent study on the Swedish BioFINDER cohort has investigated the level of plasma NfL in 13 NDDs, highlighting that NfL concentration is considerably increased in all cortical neurodegenerative disorders, amyotrophic lateral sclerosis (ALS), and atypical parkinsonian disorders, allowing the discrimination of neurodegenerative disorders from psychiatric disorders.[73] Several studies rely on in-house developed assays or commercial "for research use only" ELISAs for NfL measurements, although the limit of quantification of most of them is insufficient to measure clinically relevant NfL levels in CSF of control patients or in blood samples of patients affected by ALS.[74] In recent years, although novel ultrasensitive technologies have been employed for NfL quantification in CSF and blood, no reference method is available. NfL wider implementation in clinical practice and its use in clinical trials call for the development of a standardization program and of reference materials.[75]

The EMPIR project NeuroMET2 has allowed the creation of a primary calibrator for NfL characterized by purity assessment of the intact protein and by amino acid analysis to assign a value to the mass fraction. Even though the validation of a candidate RMP is still in progress, antibody-free and immunoprecipitation-based strategies have been developed to reach the required sensitivity for the detection of NfL in clinical CSF samples. This work has also been supported by the MIRIADE consortium.[76] Nonetheless, there is a strong need for the development of an MS-based candidate RMP method to be used to assign values to potential CRMs and establish a correlation with immunoassays. NfL levels in clinical specimens need to be correlated to patient data such as age, sex, disease onset, BMI, and kidney function. Some of these parameters have shown correlation to NfL levels in previous studies and need further investigation through the use of reference materials, thus facilitating the establishment of relevant clinical thresholds.[77,78]

A commutability study was organized to identify the most suitable candidate reference materials for NfL in blood.[79] Four analytical platforms (Simoa, Olink, Ella, and Atellica) were employed for the analysis of 40 clinical samples, each with paired serum and plasma, and seven candidate RMs, consisting of serum or plasma, spiked with either recombinant human NfL or CSF. Taken together the results show that the strong correlations among the different methods indicate a similar selectivity and that the bias could be due to the use of different calibration materials. Moreover, serum resulted in the most promising matrix for the production of CRM and spiking with CSF rather than with the recombinant protein gave the best results. The study highlights the need for RMPs and CRMs for this promising biomarker. A recent study suggests that NfL in CSF is a truncated dimer,[80] which may result in immunoassays and MS-based methods being capable of measuring different absolute concentrations of the biomarker. Characterizing the exact nature of the biomarker in biofluids will be important if full standardization of different methods is to be achieved.

7 | CONCLUSIONS

In the last 20 years important efforts have been devoted to the standardization/harmonization of fluid biomarkers for AD. The availability of high-performing certified assays will allow the definition of biomarker cutoff values and of patients' stratification criteria. Clear routes to standardization are built through the tight cooperation of national metrology institutes, clinicians, researchers, and IVD providers. RMPs and CRMs are now available for Aβ and referenced in the JCTLM database. A candidate RMP for t-tau has been recently published and will be submitted to JCTLM as well as candidate CRMs after the completion of the commutability study which is ongoing. Although several LC-MS methods have been developed for multiple p-tau phosphoforms, they are still under investigation to estimate their suitability for becoming RMPs. NfL is an emerging biomarker recently included in the AT(N) framework for which RMPs and CRMs are under development.

These standardization efforts will hopefully facilitate the regulatory approval of novel IVD devices, most being commercialized as "research use only": this collaborative work will contribute to gain improved knowledge of their performance by providing correlation with reference materials and potentially allowing an in-silico recalibration of immunoassays. This will help IVD providers to address the traceability requirements of the In Vitro Diagnostic Regulation (IVDR)[81] and is in line with the priorities of the EMN-Traceability in Laboratory Medicine which is committed to the development of SI-traceable IVD medical devices to underpin their regulatory approval by ensuring compliance to the IVDR.

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CONFLICT OF INTEREST STATEMENT

K.B. has served as a consultant, on advisory boards, or on data monitoring committees for Abcam, Axon, BioArctic, Biogen, JOMDD/Shimadzu, Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, PharmatrophiX, Prothena, 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, outside the work presented in this paper. H.Z. has served at scientific advisory boards and/or as a consultant for AbbVie, Alector, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, NervGen, Novo Nordisk, Passage Bio, Pinteon Therapeutics, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, AlzeCure, Biogen, and Roche, 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). The remaining authors (C.G., V.D., U.A., and J.G.) have no conflicts of interest to declare.Author disclosures are available in the supporting information.

CONSENT STATEMENT

As the paper is a literature overview, consent was not necessary.

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REFERENCES

1. McDade E, Cummings JL, Dhadda S, et al. Lecanemab in patients with early Alzheimer's disease: detailed results on biomarker, cognitive, and clinical effects from the randomized and open-label extension of the phase 2 proof-of-concept study. *Alzheimers Res Ther*. 2022;14(1):191. doi:10.1186/s13195-022-01124-2
2. Mintun MA, Lo AC, Duggan Evans C, et al. Donanemab in early Alzheimer's disease. *N Engl J Med*. 2021;384(18):1691-1704. doi:10.1056/NEJMoa2100708
3. Swanson CJ, Zhang Y, Dhadda S, et al. A randomized, double-blind, phase 2b proof-of-concept clinical trial in early Alzheimer's disease with lecanemab, an anti-Aβ protofibril antibody [published correction appears in Alzheimers Res Ther. 2022 May 21;14(1):70]. *Alzheimers Res Ther*. 2021;13(1):80. doi:10.1186/s13195-021-00813-8
4. van Dyck CH, Swanson CJ, Aisen P, et al. Lecanemab in early Alzheimer's disease. *N Engl J Med*. 2023;388(1):9-21. doi:10.1056/NEJMoa2212948
5. Chávez-Fumagalli MA, Shrivastava P, Aguilar-Pineda JA, et al. Diagnosis of Alzheimer's disease in developed and developing countries: systematic review and meta-analysis of diagnostic test accuracy. *J Alzheimers Dis Rep*. 2021;5(1):15-30. doi:10.3233/ADR-200263. Published 2021 Jan 11.
6. Khan TK, Alkon DL. Alzheimer's disease cerebrospinal fluid and neuroimaging biomarkers: diagnostic accuracy and relationship to drug efficacy. *J Alzheimers Dis*. 2015;46(4):817-836. doi:10.3233/JAD-150238
7. Blennow K, Zetterberg H. Biomarkers for Alzheimer's disease: current status and prospects for the future. *J Intern Med*. 2018;284(6):643-663. doi:10.1111/joim.12816
8. Gille B, Dedeene L, Stoops E, et al. Automation on an open-access platform of Alzheimer's disease biomarker immunoassays. *SLAS Technol*. 2018;23(2):188-197. doi:10.1177/2472630317750378
9. Jack CR Jr, Bennett DA, Blennow K, et al. NIA-AA Research Framework: toward a biological definition of Alzheimer's disease. *Alzheimers Dement*. 2018;14(4):535-562. doi:10.1016/j.jalz.2018.02.018
10. Dubois B, Feldman HH, Jacova C, et al. Advancing research diagnostic criteria for Alzheimer's disease: the IWG-2 criteria [published correction appears in Lancet Neurol. 2014 Aug;13(8):757]. *Lancet Neurol*. 2014;13(6):614-629. doi:10.1016/S1474-4422(14)70090-0
11. Dubois B, Feldman HH, Jacova C, et al. Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. *Lancet Neurol*. 2007;6(8):734-746. doi:10.1016/S1474-4422(07)70178-3
12. Sperling RA, Aisen PS, Beckett LA, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7(3):280-292. doi:10.1016/j.jalz.2011.03.003
13. Albert MS, DeKosky ST, Dickson D, et al. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7(3):270-279. doi:10.1016/j.jalz.2011.03.008
14. McKhann GM, Knopman DS, Chertkow H, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7(3):263-269. doi:10.1016/j.jalz.2011.03.005
15. Dubois B, Hampel H, Feldman HH, et al. Preclinical Alzheimer's disease: Definition, natural history, and diagnostic criteria. *Alzheimers Dement*. 2016;12(3):292-323. doi:10.1016/j.jalz.2016.02.002
16. Mattsson-Carlgren N, Andersson E, Janelidze S, et al. Aβ deposition is associated with increases in soluble and phosphorylated tau that precede a positive Tau PET in Alzheimer's disease. *Sci Adv*. 2020;6(16):eaaz2387. doi:10.1126/sciadv.aaz2387
17. Ebenau JL, Timmers T, Wesselman LMP, et al. ATN classification and clinical progression in subjective cognitive decline: the SCIENCe project. *Neurology*. 2020;95(1):e46-e58. doi:10.1212/WNL.0000000000009724
18. Delmotte K, Schaeverbeke J, Poesen K, Vandenberghe R. Prognostic value of amyloid/tau/neurodegeneration (ATN) classification based on diagnostic cerebrospinal fluid samples for Alzheimer's disease. *Alzheimers Res Ther*. 2021;13(1):84. doi:10.1186/s13195-021-00817-4
19. Grøntvedt GR, Lauridsen C, Berge G, et al. The amyloid, tau, and neurodegeneration (A/T/N) classification applied to a clinical research

cohort with long-term follow-up. *J Alzheimers Dis*. 2020;74(3):829-837. doi:10.3233/JAD-191227
20. Petersen RC, Aisen P, Boeve BF, et al. Mild cognitive impairment due to Alzheimer disease in the community. *Ann Neurol*. 2013;74(2):199-208. doi:10.1002/ana.23931
21. Dementi Scheltens. Mild cognitive impairment–amyloid and beyond. *Nat Rev Neurol*. 2013;9(9):493-495. doi:10.1038/nrneurol.2013.147
22. Abner EL, Kryscio RJ, Schmitt FA, et al. Outcomes after diagnosis of mild cognitive impairment in a large autopsy series. *Ann Neurol*. 2017;81(4):549-559. doi:10.1002/ana.24903
23. Chételat G. Alzheimer disease: aβ-independent processes-rethinking preclinical AD. *Nat Rev Neurol*. 2013;9(3):123-124. doi:10.1038/nrneurol.2013.21
24. Jack CR Jr, Knopman DS, Jagust WJ, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol*. 2010;9(1):119-128. doi:10.1016/S1474-4422(09)70299-6
25. Oberstein TJ, Schmidt MA, Florvaag A, et al. Amyloid-β levels and cognitive trajectories in non-demented pTau181-positive subjects without amyloidopathy. *Brain*. 2022;145(11):4032-4041. doi:10.1093/brain/awac297
26. Pascoal TA, Leuzy A, Therriault J, et al. Discriminative accuracy of the A/T/N scheme to identify cognitive impairment due to Alzheimer's disease. *Alzheimers Dement (Amst)*. 2023;15(1):e12390. doi:10.1002/dad2.12390
27. Rosenberg A, Öhlund-Wistbacka U, Hall A, et al. Neurodegeneration classification and eligibility for anti-amyloid treatment in a memory clinic population. *Neurology*. 2022;99(19):e2102-e2113. doi:10.1212/WNL.0000000000201043
28. Hansson O, Rutz S, Zetterberg H, et al. Pre-analytical protocol for measuring Alzheimer's disease biomarkers in fresh CSF [published correction appears in Alzheimers Dement (Amst). 2021 Apr 09;13(1):e12176]. *Alzheimers Dement (Amst)*. 2020;12(1):e12137. doi:10.1002/dad2.12137
29. Willemse EAJ, Tijms BM, van Berckel BNM, et al. Comparing CSF amyloid-beta biomarker ratios for two automated immunoassays, Elecsys and Lumipulse, with amyloid PET status. *Alzheimers Dement (Amst)*. 2021;13(1):e12182. doi:10.1002/dad2.12182
30. Cousins KAQ, Phillips JS, Irwin DJ, et al. ATN incorporating cerebrospinal fluid neurofilament light chain detects frontotemporal lobar degeneration. *Alzheimers Dement*. 2021;17(5):822-830. doi:10.1002/alz.12233
31. Quaglia M, Bellotti V, Cano S, et al. Better measurement for improved diagnosis and management of alzheimer's disease: update on the empir neuromet project. *Alzheimer's Dement*. 2018;14:P759-P760. doi:10.1016/j.jalz.2018.06.920
32. Quaglia M, Cano S, Fillmer A, et al. The NeuroMET project: metrology and innovation for early diagnosis and accurate stratification of patients with neurodegenerative diseases. *Alzheimer's Dement*. 2021;17:e053655. doi:10.1002/alz.053655
33. ISO 17511:2020. In vitro diagnostic medical devices — Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples
34. Miller WG, Greenberg N. Harmonization and Standardization: where are we now. *J Appl Lab Med*. 2021;6(2):510-521. doi:10.1093/jalm/jfaa189
35. Joseph Ralf, G Martos, et al.Establishment of measurement traceability for peptide and protein quantification through rigorous purity assessment—a review 2019 Metrologia 56 044006. doi:10.1088/1681-7575/ab27e5
36. Rozanova S, Barkovits K, Nikolov M, Schmidt C, Urlaub H, Marcus K. Quantitative mass spectrometry-based proteomics: an overview. *Methods Mol Biol*. 2021;2228:85-116. doi:10.1007/978-1-0716-1024-4_8
37. Calderón-Celis F, Encinar JR. Sanz-Medel A. Standardization approaches in absolute quantitative proteomics with mass spectrometry. *Mass Spectrom Rev*. 2018;37(6):715-737. doi:10.1002/mas.21542
38. https://www.bipm.org/documents/20126/2071204/JCGM_100_2008_E.pdf/cb0ef43f-baa5-11cf-3f85-4dcd86f77bd6
39. Amft M, Ortner M, Eichenlaub U, et al. The cerebrospinal fluid biomarker ratio Aβ42/40 identifies amyloid positron emission tomography positivity better than Aβ42 alone in a heterogeneous memory clinic cohort. *Alzheimers Res Ther*. 2022;14(1):60. doi:10.1186/s13195-022-01003-w
40. Korecka M, Waligorska T, Figurski M, et al. Qualification of a surrogate matrix-based absolute quantification method for amyloid-β_{42} in human cerebrospinal fluid using 2D UPLC-tandem mass spectrometry. *J Alzheimers Dis*. 2014;41(2):441-451. doi:10.3233/JAD-132489
41. Leinenbach A, Pannee J, Dülffer T, et al. Mass spectrometry-based candidate reference measurement procedure for quantification of amyloid-β in cerebrospinal fluid. *Clin Chem*. 2014;60(7):987-994. doi:10.1373/clinchem.2013.220392
42. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Validation of analytical procedures: text and methodology Q2(R1). ICH harmonised tripartite guideline. Current Step 4 version. 2005. (Accessed-February 2014). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf
43. *International Organization for Standardization. ISO guide 15193. In vitro diagnostic medical devices: measurement of quantities in samples of biological origin; requirements for content and presentation of reference measurement procedures*. ISO, 2009
44. Korecka M, Figurski MJ, Landau SM, et al. Analytical and clinical performance of amyloid-beta peptides measurements in CSF of ADNIGO/2 participants by an LC-MS/MS reference method. *Clin Chem*. 2020;66(4):587-597. doi:10.1093/clinchem/hvaa012
45. Pannee J, Gobom J, Shaw LM, et al. Round robin test on quantification of amyloid-β 1-42 in cerebrospinal fluid by mass spectrometry. *Alzheimers Dement*. 2016;12(1):55-59. doi:10.1016/j.jalz.2015.06.1890
46. Kuhlmann J, Andreasson U, Pannee J, et al. CSF Aβ1-42 - an excellent but complicated Alzheimer's biomarker - a route to standardisation. *Clin Chim Acta*. 2017;467:27-33. doi:10.1016/j.cca.2016.05.014
47. Bjerke M, Andreasson U, Kuhlmann J, et al. Assessing the commutability of reference material formats for the harmonization of amyloid-β measurements. *Clin Chem Lab Med*. 2016;54(7):1177-1191. doi:10.1515/cclm-2015-0733
48. Andreasson U, Kuhlmann J, Pannee J, et al. Commutability of the certified reference materials for the standardization of β-amyloid 1-42 assay in human cerebrospinal fluid: lessons for tau and β-amyloid 1-40 measurements. *Clin Chem Lab Med*. 2018;56(12):2058-2066. doi:10.1515/cclm-2018-0147
49. Boulo S, Kuhlmann J, Andreasson U, et al. First amyloid β1-42 certified reference material for re-calibrating commercial immunoassays. *Alzheimers Dement*. 2020;16(11):1493-1503. doi:10.1002/alz.12145
50. Verberk IMW, Misdorp EO, Koelewijn J, et al. Characterization of pre-analytical sample handling effects on a panel of Alzheimer's disease-related blood-based biomarkers: results from the Standardization of Alzheimer's Blood Biomarkers (SABB) working group. *Alzheimers Dement*. 2022;18(8):1484-1497. doi:10.1002/alz.12510
51. Pannee J, Shaw LM, Korecka M, et al. The global Alzheimer's Association round robin study on plasma amyloid β methods. *Alzheimers Dement (Amst)*. 2021;13(1):e12242. doi:10.1002/dad2.12242
52. Barthélemy NR, Bateman RJ, Hirtz C, et al. Cerebrospinal fluid phospho-tau T217 outperforms T181 as a biomarker for the differential diagnosis of Alzheimer's disease and PET amyloid-positive patient identification. *Alzheimers Res Ther*. 2020;12(1):26. doi:10.1186/s13195-020-00596-4
53. Horie K, Barthélemy NR, Mallipeddi N, et al. Regional correlation of biochemical measures of amyloid and tau phosphorylation in the brain.

Acta Neuropathol Commun. 2020;8(1):149. doi:10.1186/s40478-020-01019-z
54. Gobom J, Benedet AL, Mattsson-Carlgren N, et al. Antibody-free measurement of cerebrospinal fluid tau phosphorylation across the Alzheimer's disease continuum. *Mol Neurodegener*. 2022;17(1):81. doi:10.1186/s13024-022-00586-0
55. Milà-Alomà M, Ashton NJ, Shekari M, et al. Plasma p-tau231 and p-tau217 as state markers of amyloid-β pathology in preclinical Alzheimer's disease [published correction appears in Nat Med. *Nat Med*. 2022;28(9):1797-1801. doi:10.1038/s41591-022-01925-w
56. Cullen NC, Leuzy A, Janelidze S, et al. Plasma biomarkers of Alzheimer's disease improve prediction of cognitive decline in cognitively unimpaired elderly populations. *Nat Commun*. 2021;12(1):3555. doi:10.1038/s41467-021-23746-0
57. Bayoumy S, Verberk IMW, den Dulk B, et al. Clinical and analytical comparison of six Simoa assays for plasma P-tau isoforms P-tau181, P-tau217, and P-tau231. *Alzheimers Res Ther*. 2021;13(1):198. doi:10.1186/s13195-021-00939-9
58. Janelidze S, Bali D, Ashton NJ, et al. Head-to-head comparison of 10 plasma phospho-tau assays in prodromal Alzheimer's disease. *Brain*. 2022;333. doi:10.1093/brain/awac333. published online ahead of print, 2022 Sep 10.
59. Delaby C, Teunissen CE, Blennow K, et al. Clinical reporting following the quantification of cerebrospinal fluid biomarkers in Alzheimer's disease: an international overview. *Alzheimers Dement*. 2022;18(10):1868-1879. doi:10.1002/alz.12545
60. Korecka M, Shaw LM. Mass spectrometry-based methods for robust measurement of Alzheimer's disease biomarkers in biological fluids. *J Neurochem*. 2021;159(2):211-233. doi:10.1111/jnc.15465
61. Kang JH, Korecka M, Toledo JB, Trojanowski JQ, Shaw LM. Clinical utility and analytical challenges in measurement of cerebrospinal fluid amyloid-β(1-42) and τ proteins as Alzheimer disease biomarkers. *Clin Chem*. 2013;59(6):903-916. doi:10.1373/clinchem.2013.202937
62. McAvoy T, Lassman ME, Spellman DS, et al. Quantification of tau in cerebrospinal fluid by immunoaffinity enrichment and tandem mass spectrometry. *Clin Chem*. 2014;60(4):683-689. doi:10.1373/clinchem.2013.216515
63. Bros P, Vialaret J, Barthelemy N, et al. Antibody-free quantification of seven tau peptides in human CSF using targeted mass spectrometry. *Front Neurosci*. 2015;9:302. doi:10.3389/fnins.2015.00302
64. Barthélemy NR, Gabelle A, Hirtz C, et al. Differential mass spectrometry profiles of tau protein in the cerebrospinal fluid of patients with Alzheimer's disease, progressive supranuclear palsy, and dementia with lewy bodies. *J Alzheimers Dis*. 2016;51(4):1033-1043. doi:10.3233/JAD-150962
65. Viodé A, Epelbaum S, Benyounes I, et al. Simultaneous quantification of tau and α-synuclein in cerebrospinal fluid by high-resolution mass spectrometry for differentiation of Lewy Body dementia from Alzheimer's Disease and controls. *Analyst*. 2019;144(21):6342-6351. doi:10.1039/c9an00751b
66. Pottiez G, Yang L, Stewart T, et al. Mass-spectrometry-based method to quantify in parallel tau and amyloid β 1-42 in CSF for the diagnosis of Alzheimer's disease. *J Proteome Res*. 2017;16(3):1228-1238. doi:10.1021/acs.jproteome.6b00829
67. Zhou M, Haque RU, Dammer EB, et al. Targeted mass spectrometry to quantify brain-derived cerebrospinal fluid biomarkers in Alzheimer's disease. *Clin Proteomics*. 2020;17:19. doi:10.1186/s12014-020-09285-8
68. Giangrande C, Vaneeckhoutte H, Boeuf A, et al. Development of a candidate reference measurement procedure by ID-LC-MS/MS for total tau protein measurement in cerebrospinal fluid (CSF). *Clin Chem Lab Med*. 2023:2022-1250. doi:10.1515/cclm-2022-1250. published online ahead of print, 2023 Feb 24.
69. Mattsson N, Zetterberg H, Janelidze S, et al. Plasma tau in Alzheimer disease. *Neurology*. 2016;87(17):1827-1835. doi:10.1212/WNL.0000000000003246
70. Friede RL, Samorajski T. Axon caliber related to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. *Anat Rec*. 1970;167(4):379-387. doi:10.1002/ar.1091670402
71. Khalil M, Teunissen CE, Otto M, et al. Neurofilaments as biomarkers in neurological disorders. *Nat Rev Neurol*. 2018;14(10):577-589. doi:10.1038/s41582-018-0058-z
72. Mattsson N, Cullen NC, Andreasson U, Zetterberg H, Blennow K. Association between longitudinal plasma neurofilament light and neurodegeneration in patients with Alzheimer disease. *JAMA Neurol*. 2019;76(7):791-799. doi:10.1001/jamaneurol.2019.0765. published correction appears in JAMA Neurol. 2019 Jun 10;:.
73. Ashton NJ, Janelidze S, Al Khleifat A, et al. A multicentre validation study of the diagnostic value of plasma neurofilament light. *Nat Commun*. 2021;12(1):3400. doi:10.1038/s41467-021-23620-z
74. Poesen K, Van Damme P. Diagnostic and prognostic performance of neurofilaments in ALS. *Front Neurol*. 2019;9:1167. doi:10.3389/fneur.2018.01167
75. Forgrave LM, Ma M, Best JR, DeMarco ML. The diagnostic performance of neurofilament light chain in CSF and blood for Alzheimer's disease, frontotemporal dementia, and amyotrophic lateral sclerosis: a systematic review and meta-analysis. *Alzheimers Dement (Amst)*. 2019;11:730-743. doi:10.1016/j.dadm.2019.08.009
76. Mavrina E, Kimble L, Waury K, et al. Multi-omics interdisciplinary research integration to accelerate dementia biomarker development (MIRIADE). *Front Neurol*. 2022;13:890638. doi:10.3389/fneur.2022.890638
77. Hermesdorf M, Leppert D, Maceski A, et al. Longitudinal analyses of serum neurofilament light and associations with obesity indices and bioelectrical impedance parameters. *Sci Rep*. 2022;12(1):15863. doi:10.1038/s41598-022-20398-y
78. Akamine S, Marutani N, Kanayama D, et al. Renal function is associated with blood neurofilament light chain level in older adults. *Sci Rep*. 2020;10(1):20350. doi:10.1038/s41598-020-76990-7
79. Andreasson U, Gobom J, Delatour V, et al. Assessing the commutability of candidate reference materials for the harmonization of neurofilament light measurements in blood. *Clin Chem Lab Med*. 2023. doi:10.1515/cclm-2022-1181. published online ahead of print, 2023 Jan 31.
80. Meda FJ, Knowles K, Swift IJ, et al. Neurofilament light oligomers in neurodegenerative diseases: quantification by homogeneous immunoassay in cerebrospinal fluid. *BMJ Neurol Open*. 2023;5(1):e000395. doi:10.1136/bmjno-2022-000395
81. https://eur-lex.europa.eu/eli/reg/2017/746/oj

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