CEREBROSPINAL FLUID BIOMARKERS

An automated clinical mass spectrometric method for identification and quantification of variant and wild-type amyloid-\(\beta\) 1-40 and 1-42 peptides in CSF

Mari L. DeMarco\(^1\), Quyen Nguyen\(^2\), Alice Fok\(^3\), Ging-Yuek Robin Hsiung\(^3\), J. Grace van der Gugten\(^1\)

\(^1\)Department of Pathology and Laboratory Medicine, Providence Health Care, Vancouver, British Columbia, Canada
\(^2\)Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada
\(^3\)Division of Neurology, University of British Columbia, Vancouver, British Columbia, Canada

Correspondence
Mari L. DeMarco, St. Paul’s Hospital, 1081 Burrard St, Vancouver, BC, Canada V6Z 1Y6. E-mail: mdeMarco@mail.ubc.ca

Abstract

Introduction: We developed an automated liquid chromatography-tandem mass spectrometry high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for multiplex quantification of wild-type (wt) amyloid \(\beta\) (A\(\beta\)) peptides 1-40 (A\(\beta\)40) and 1-42 (A\(\beta\)42) and detection of variant A\(\beta\) peptides in cerebrospinal fluid.

Methods: The multiplex A\(\beta\) HPLC-MS/MS assay was validated in a clinically accredited laboratory following regulatory guidelines, with A\(\beta\)42 calibration assigned to the ERM/IFCC certified reference material; sequence variants were additionally multiplexed into the method.

Results: Sample preparation was fully automated on a liquid handler. The assay quantified wt-A\(\beta\)42 and wt-A\(\beta\)40 and detected sequence variants, when present, within the A\(\beta\)42 sequence.

Discussion: Extension of the HPLC-MS/MS approach for quantification of wt-A\(\beta\)42 and wt-A\(\beta\)40 to include known sequence variants increases analytical accuracy of the mass spectrometric approach and enables identification of cases of autosomal dominant Alzheimer’s disease. Development of an automated workflow and selection of appropriate instrumentation enabled deployment of this method in routine clinical testing.

KEYWORDS
Alzheimer’s disease, amyloid-\(\beta\) peptides, automation, autosomal dominant Alzheimer’s disease, cerebrospinal fluid, certified reference material, mass spectrometry, variant

1 | INTRODUCTION

As a biomarker of amyloid pathology, the concentration of amyloid \(\beta\) (A\(\beta\)) peptides in cerebrospinal fluid (CSF) is used in the ante-mortem diagnosis of Alzheimer’s disease (AD). In CSF, a reduction in A\(\beta\) concentration—specifically the 1-42 residue isoform A\(\beta\)42—is highly correlated with AD, and associated with the sequestration of A\(\beta\)42 in extracellular aggregates in the brain.\(^1\) Confirmatory
diagnosis of AD can only be made by identification of Aβ aggregates in brain tissue (most commonly by post-mortem analysis), with the exception of autosomal dominant forms of the disease, which can be identified by DNA sequencing. Aβ peptides arise from cleavage of the trans-membrane amyloid-precursor protein (APP) by endogenous proteases, resulting in peptides of various lengths, including residues 1-40 and 1-42, referred to herein as Aβ40 and Aβ42, respectively (Figure 1). Genetic forms of AD (ie, autosomal dominant AD) include highly penetrant pathogenic sequence variants within the APP gene and two presenilin genes (PSEN1 and PSEN2). The term "mutation" was previously used to describe such sequence variants, but has been replaced with "variant" further annotated with its pathogenicity or lack thereof.

As the ante-mortem diagnosis of AD moves from one identifying clinical syndromes to a biochemical definition reflective of the pathological accumulation of amyloid plaques and neurofibrillary tangles, methods for accurate, precise, and selective quantification of these biomarkers gains greater importance. Historically, Aβ in CSF has been measured predominantly via immunoassay; however, immunoassays for Aβ have demonstrated high intra- and inter-laboratory variability, which spurred the development of alternate approaches, namely automated immunoassays and high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). With HPLC-MS/MS methods, intra- and inter-laboratory precision were improved; however, variability associated with multiple operators, which would occur in a clinical laboratory setting, remained unaddressed.

An advantage of HPLC-MS/MS is exquisite analyte selectivity derived from the use of multiple reaction monitoring (MRM). HPLC is used to separate analytes in time, and MS/MS enables further selectivity by the analyte's mass-to-charge (m/z) ratio and subsequent confirmation of the analyte's primary structure (ie, amino acid sequence) by fragmentation. The use of MRM is akin to performing a sequencing experiment in which the peptide (ie, precursor ion) is reproducibly fragmented into a series of overlapping peptide sequences, and the fragment m/z ratios are then detected to confirm the identity of the target analyte. This level of selectivity, far exceeding that of immunoassays, is the reason the laboratory medicine community has turned to mass spectrometry for the development of clinical reference methods and for routine quantification of clinically relevant peptide and proteins, including a candidate reference method for Aβ.

The selectivity of HPLC-MS/MS, however, is a double-edged sword in that MRM methods are so selective they ignore all peptides except those satisfying the MRM criteria, that is, an analyte (1) eluting at a specific time from the analytical column, and (2) having the predefined MRM transitions. Early/late eluting peptides are ignored by the method, as are those not meeting the m/z transition criteria—as would occur if there were an alteration to the Aβ sequence. Currently published mass spectrometric methods, including those using in vitro proteolytic digestion, do not include MRMs of known Aβ sequence variants and due to the high analytical selectivity of the MRM approach, Aβ variants are not "seen" by the method. Immunoassays, for their part, cannot differentiate between Aβ variants that occur outside of the assay epitope regions, or if the amino acid change does occur within the epitope regions it may abrogate binding/detection.

### RESEARCH IN CONTEXT

1. Systematic review: Development of an automated and multiplex wild-type/variant amyloid β (Aβ40 and Aβ42) high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) assay that supports diagnostic workup of sporadic AD and can identify autosomal dominant AD via detection of amyloid-precursor protein (APP) pathogenic variants within the Aβ sequence.

2. Interpretation: The method enables ease of operation in a clinical setting via automation and use of equipment commonly found in clinical laboratories.

3. Future directions: With the availability of a highly selective tool that detects Aβ variants, such cases can be investigated with greater resolution (eg, temporal changes in peptide isoform concentrations) to further our understanding of disease progression and pathology.

### METHODS

#### 2.1 Samples

With research ethics board approval, human CSF was collected from patients at the University of British Columbia’s Clinic for Alzheimer’s Disease and Related Disorders and at St. Paul’s Hospital, in Vancouver, Canada. Specimens were collected by a standardized protocol,
FIGURE 1 The transmembrane amyloid precursor protein (APP) is cleaved by β- and γ-secretase to form the 1-40 and 1-42 residue isoforms of amyloid beta (Aβ). In addition to proteolytic isoforms, there are known amino acid variants, both pathogenic and benign, within the Aβ42 sequence including collection directly into polypropylene tubes. Diagnostic classification was based on thorough clinical assessment by a neurologist with expertise in neurodegenerative disorders.

2.2 Sample preparation and HPLC-MS/MS analysis

A detailed description of the HPLC-MS/MS method (Tables S1-S3 in supporting information), including material sources, can be found in the supporting information. In brief, 200 μL of each sample (ie, CSF specimens, quality controls [QC], and calibrators) was treated with guanidine hydrochloride (GdnHCl) containing 15N-Aβ40 and 15N-Aβ42 internal standards (ISs), subjected to reversed phase solid-phase extraction (SPE), and eluted into a 96-well plate. These steps were performed either manually or using a liquid handling robot. Analysis was performed using a C18 analytical column coupled to a triple quadrupole mass spectrometer.

2.3 Validation

Method validation was performed following the Clinical and Laboratory Standards Institute (CLSI) guidelines including C62, EP-5A, and EP-6A. Validation experiments included assessments of:

- (1) recovery and linearity,
- (2) ion suppression and enhancement,
- (3) precision, and
- (4) quality measures—including determination of the analytical measuring interval (AMI), clinical measuring interval (CMI), lower limit of the measuring interval (LLMI), and method comparisons.

2.3.1 Recovery and linearity

Linearity was assessed via a mixing study, following CLSI EP6-A, using a “low” concentration human CSF pool (Aβ40 = 1067 ng/L; Aβ42 = 220 ng/L) and a “high” pool (Aβ40 = 24067 ng/L; Aβ42 = 3387 ng/L), which was made by supplementing a human CSF pool with synthetic Aβ peptides.

2.3.2 Ion suppression and enhancement

Ion suppression and enhancement can result from other compounds in a complex sample matrix altering the ionization efficiency of the ions of interest. Ion suppression and enhancement was assessed by post-column continuous infusion, an extracted CSF pool (without the addition of IS) was injected into the LC stream and a solution containing 15N-Aβ40 and 15N-Aβ42 was directly infused into the flow at the
source via at-union. Additionally, phospholipid MRMs were monitored in extracted CSF samples in the validation phase.

### 2.3.3 | Precision

Precision experiments were performed using a modification of the CLSI EP-5A protocol: specifically, quintuplicate measurements over 5 days using human CSF pools. Three CSF pools at low, medium, and high concentrations were assessed. The mean concentration in the medium CSF pool (composed solely of human CSF) was 3900 ng/L of Aβ40 and 522 ng/L of Aβ42. The low pool (Aβ40 = 2020 ng/L; Aβ42 = 273 ng/L) was made by diluting the medium pool with the artificial CSF and the high pool (Aβ40 = 10100 ng/L; Aβ42 = 1288 ng/L) was made by supplementing the medium pool with synthetic Aβ40 and Aβ42.

### 2.3.4 | Accuracy and method comparison

Wt-Aβ42 calibrators were assigned to the ERM/IFCC certified reference material (CRM), and wt-Aβ40 was calibrated against peptide-manufacturer reported mass, adjusted for HPLC purity. For wt-Aβ42 calibrator assignment, three CRMs—ERMA480/IFCC, ERM-DA481/IFCC, ERM-DA482/IFCC—were run in duplicate (within run) and in three batches (between run) with the average concentrations set to the concentrations from the certificates of analyses. The calibrators were run as unknowns in the three batches and the mean of the observed concentrations used for assignment. A regression analysis was performed by weighted Deming regression between the CRM-assigned calibrator concentrations and the pre-CRM-assigned concentrations (ie, manufacturer stated amino acid analysis and HPLC purity).

A method comparison was performed between the HPLC-MS/MS and the INNOTEST enzyme-linked immunosorbent assay (ELISA) β-Amyloid [1-42] (n = 155 specimens).

### 2.3.5 | LLMI, AMI, and CMI

Acceptable LLMI criteria was defined by an imprecision <20% and a signal-to-noise (S/N) > 10. The AMI is the range of analyte values that a method can directly measure without modification (eg, dilution). The CMI is defined as the range of analyte values a method can measure allowing for specimen dilution, concentration, or other pre-treatment used to extend the AMI.

### 2.3.6 | Manual versus automated sample preparation

To assess the precision of the manual and automated sample preparation methods for HPLC-MS/MS analysis, human CSF samples (n = 40 individuals) with concentrations ranging between 839–10023 ng/L for Aβ40 and 113–1266 ng/L for Aβ42 were analyzed using each method.

### 2.3.7 | Variants within the Aβ42 sequence

A list of known amino acid variants within the Aβ42 sequence was developed by searching existing databases including: Alzforum (http://www.alzforum.org/mutations), Single Nucleotide Polymorphism Database (dbSNP, NCBI), and Exome Aggregation Consortium (ExAC). A variant within the Aβ42 sequence was included in the MRM method if it satisfied the following criteria: (1) contained a variant within the Aβ42 sequence, and (2) was either pathogenic (average frequency not considered in this case), or, if non-pathogenic or of unknown significance, had an average frequency in the global population of ≥4.0E-05.

To test the wt/var-Aβ HPLC-MS/MS assay, CSF representative of the complex scenario in which an individual has one wild-type and one variant APP allele was assessed. To create a series of CSF samples characteristic of this heterozygote state, synthetic Aβ40 variants were spiked into a human CSF pool (containing only wt-Aβ) at equimolar concentration to the endogenous wt-Aβ40 (0.157 nM).

### 2.4 | Diagnostic performance

Diagnostic performance was assessed using CSF from 93 individuals presenting with cognitive complaints to a memory clinic. All individuals were evaluated by a standardized protocol with diagnosis made by clinical assessment: probable AD (n = 39) and cognitive complaints/impairment due to a non-AD cause (n = 54). In addition to use of the wt/var-Aβ HPLC-MS/MS assay, total tau was quantified by ELISA (INNOTEST hTau Ag) and data analyzed using receiver operating characteristic (ROC) curves.

### 2.5 | Data analysis

Instrument data were viewed and analyzed using Analyst software (SCIEX v.1.6), Excel (Microsoft), and R (v.3.4.0).

### 3 | RESULTS

#### 3.1 | Figures of merit

#### 3.1.1 | Recovery and linearity

The method was linear from at least 100–20,000 ng/L for Aβ40 and 100–3000 ng/L for Aβ42 (Figure S1 and Table S4-S5 in supporting information).

#### 3.1.2 | Ion suppression and enhancement

There was no observable ion suppression or enhancement occurring at the retention time of the Aβ40 and Aβ42 peptides, or due to phospholipids (data not shown).
FIGURE 2 Manual and automated sample preparation prior to high performance liquid chromatography tandem mass spectrometry analysis demonstrated comparable performance for both (A and B) amyloid β 1-42 (Aβ42) and (C and D) amyloid β 1-40 (Aβ40). A and C, Shaded region represents the 95% confidence interval (CI) of the slope of the linear regression. B and D, Black dashed lines represent the 95% CI of the mean difference (solid line).

3.1.3 | Precision and automated versus manual sample preparation

Intra- and inter-run precision data for manual and automated sample preparation protocols can be found in Table S6 in supporting information. By regression analysis for Aβ42: $Y_{\text{automated}} = 1.01 \times X_{\text{manual}} + 17.63$, $R^2 = 0.9693$, confidence interval (CI) slope: [0.942, 1.074], CI intercept: [–70.843, 36.119] (Figure 2A). By regression analysis for Aβ40: $Y_{\text{automated}} = 0.95 \times X_{\text{manual}} + 0.06$, $R^2 = 0.9728$, CI slope: [0.895, 1], CI intercept: [–0.38, 0.297] (Figure 2C). Difference plots for the automated versus manual workflow revealed a mean bias of –1.64%, 95% CI: [–20.94, 17.66] for Aβ42 and –6.00%, 95% CI: [–22.54, 10.55] for Aβ40 (Figure 2B and D).

3.1.4 | Accuracy and method comparison for Aβ42

The CRM-assigned calibrators yielded the following regression equation to the calibrators’ pre-CRM assignment: $Y_{\text{CRM}} = 0.89 \times X_{\text{pre-CRM}} + 9.31$, $R^2 = 0.9904$, CI slope: [0.769, 1.04], CI intercept: [–10.676, 91.708] (Figure 3). The method comparison between the HPLC-MS/MS assay and the predicate ELISA method revealed the following by linear regression analysis: $Y_{\text{HPLC-MS/MS}} = 2.64 \times X_{\text{ELISA}} -247.4$, $R^2 = 0.63$, CI slope: [2.38, 2.99], CI intercept: [–398.75, –123.67] and a mean bias of 71.5%, 95% CI: [123.6, 19.4], noting that the ELISA is not calibrated to the ERM/IFCC CRM (Figure S2 in supporting information).

3.1.5 | LMI, AMI, and CMI

For the calibrator with a concentration of 100 ng/L for both Aβ40 and Aβ42, the average back-calculated concentration for Aβ40 was 106 ng/L with an average S/N of 47 and for Aβ42 was 105 ng/L with an average S/N of 23. Given the acceptable S/N ratio for both Aβ40 and Aβ42 at 100 ng/L, the reportable LLMI for both peptides was set to 100 ng/L (Figure S3 in supporting information). Due to the broad analytical range of the method no extra dilution procedures were required and thus the AMI was equivalent to the CMI at 100–20000 ng/L for Aβ40 and 100–3000 ng/L for Aβ42.

3.2 | Variants within the Aβ42 sequence

From the database search (Table S7 in supporting information), a total of 20 Aβ variants were found to meet the inclusion criteria. This included 13 pathogenic variants, 1 non-pathogenic variant, and 6 variants of unknown significance (Table S8 in supporting information). As proof of concept, nine of these variants were synthesized as var-Aβ40 peptides (given that the sequence variants were captured within residues 1-40), and used to create the “heterozygous” CSF samples (Table 1).
TABLE 1  Multiple reaction monitoring transitions and retention times of wt-Aβ40 and -Aβ40 variants analyzed in the APP heterozygosity experiments

| Variant   | Name         | Mass (Da) | Retention time (min) | Precursor ion (m/z) | Product ions (m/z) |
|-----------|--------------|-----------|----------------------|---------------------|-------------------|
|           | Wild-type    | 4329.9    | 4.06                 | 1083.0              | 1054.1, 1029.1, 1000.8 |
| L34V      | Piedmont     | 4315.8    | 3.93                 | 1079.9              | 1050.5, 1011.5, 997.4 |
| A21G      | Flemish      | 4315.8    | 3.97                 | 1079.9              | 1050.5, 1011.5, 997.4 |
| H6R       | English      | 4348.9    | 4.12                 | 1088.2              | 1058.9, 1033.9, 1005.4 |
| D7N       | Tottori      | 4328.9    | 4.14                 | 1083.0              | 1054.1, 1029.1, 1000.8 |
| E22G      | Arctic       | 4257.8    | 4.18                 | 1065.5              | 1036.2, 1011.5, 997.1 |
| E22Δ      | Osaka        | 4200.8    | 4.22                 | 1051.2              | 943.9, 914.8, 882.7 |
| E22Q      | Dutch        | 4328.9    | 4.24                 | 1083.0              | 1054.1, 1029.1, 1000.8 |
| D23N      | Iowa         | 4328.9    | 4.32                 | 1083.0              | 1054.1, 1029.1, 1000.8 |
| E22K      | Italian      | 4328.9    | 4.42                 | 1083.0              | 1054.1, 1029.1, 1000.8 |

Abbreviations: Aβ, amyloid beta; APP, amyloid-precursor protein; multiple reaction monitoring; m/z, mass-to-charge ratio; wt, wild type

By HPLC-MS/MS analysis, all Aβ variants tested were resolved chromatographically from wt-Aβ (Figure 4A-J). This included variants that shared similar transitions to wt-Aβ40: E22Q, D23N, D7N, and E22K. The E22G, D23N, and E22Q variants co-eluted, with E22G readily identifiable by its unique MRMs. E22Q and D23N shared precursor masses and transitions (as currently selected) within the prescribed m/z tolerances, and therefore these two variants could not be distinguished from one another using the chromatographic conditions described (Figure 4C and F).

3.3 | Diagnostic performance

ROC curve analysis yielded the following area under the curve (AUC) for tau/Aβ42 (0.9137), Aβ42/Aβ40 (0.8305), Aβ42 (0.8436), and Aβ40 (0.5857; Figure 5). In this cohort, the biomarker cut-points at the Youden indices were as follows: 950 ng/L for Aβ42, 450 ng/L for total tau, 0.28 for the total tau/Aβ42 ratio, and 0.12 for the Aβ42/Aβ40 ratio.

4 | DISCUSSION

There were several barriers to the deployment of an Aβ peptide assay for routine care in our health-care setting including: (1) limited selection of Aβ42 assays licensed by our national regulatory agency, and (2) no licensed Aβ40 assays. For Aβ42, only an ELISA was available, which is an undesirable format for clinical laboratories. Based on previously reported mass spectrometric Aβ assays at the time of our assay development, challenges for clinical implementation included:

1) Use of instrumentation not commonly found in clinical laboratories;
2) Manual sample preparation protocols;
3) Assays typically operated by a single experienced operator (and subsequently all figures of merit reported dependent on this single operator);
4) Lack of calibration to the certified reference material;
5) No accounting for the presence of sequence variants.

For deployment in routine clinical testing, we developed a fully automated HPLC-MS/MS method for multiplex quantification of wt-Aβ40 and wt-Aβ42 and detection of var-Aβ peptides in CSF. We utilized a liquid robotic handler, chromatography system, and mass spectrometer class commonly found in hospital laboratories. For the analytical equipment, we used HPLC (mL/min flow range) coupled to triple quadrupole MS. These systems are widely used in laboratory medicine based on their longstanding use in toxicology. Lower flow liquid chromatography systems (ie, micro- and nano-flow) and high resolution/accurate-mass mass spectrometers while common in research proteomics labs are rarely used by hospital laboratories and, thus, assays on these platforms have limited uptake. As with our small molecule clinical HPLC-MS/MS assays, we automated the sample preparation on our robotic liquid handler. The resultant precision and accuracy of the automated method was comparable to that of a single experienced operator. This allowed for implementation in a hospital laboratory where staffing varies, along with operator expertise, and where an assay cannot be assigned to a single technician.

With the availability of the wt-Aβ42 CRM, the assay was calibrated to this standard. This calibration supports efforts to standardized reporting of Aβ42 mass spectrometric assays and supports harmonization of Aβ42 assays independent of the analytical platform used. Such efforts facilitate comparisons of data (and cut-points) across different methods, instruments, and laboratories. Based on known variability in amino acid analysis and HPLC-based purity assessments and our historical lot-to-lot calibrator cross-over data, CRM assignment should be established for each new lot of peptide stocks. Aβ40 was calibrated to the peptide-manufacturer stated product mass and purity, and with future availability of a CRM, we can apply the procedure described herein to the Aβ40 calibrators as well. Comparison of the Aβ42 assay with the INNOTEST ELISA (not calibrated to the CRM), demonstrated a regression profile consistent with that
The multiplex amyloid beta (A\(\beta\)) high performance liquid chromatography tandem mass spectrometry assay enabled identification of cases (A) homozygous for wild-type amyloid-precursor protein (wt-APP) alleles and (B-J) heterozygous for an Alzheimer’s disease autosomal dominant APP variant, as demonstrated by the multiple reaction monitoring chromatograms. A. In the homozygous sample, a single wt-A\(\beta\)40 peak (blue) is observed. B-J. In the heterozygous samples, both the wt-A\(\beta\)40 (blue) and var-A\(\beta\)40 (magenta) peaks are observed, the latter corresponding to the following pathogenic variants: (B) Arctic (E22G), (C) Dutch (E22Q), (D) English (H6R), (E) Flemish (A21G), (F) Iowa (D23N), (G) Italian (E22K), (H) Osaka (E22Δ), (I) Piedmont (L34V), and (J) Tottori (D7N).

Previously noted for other HPLC-MS/MS assays with comparisons to the Luminex xMAP and INNOTEST ELISA.26,27

Corroborating previous findings, tau/A\(\beta\)42 demonstrated the highest AUC in the ROC curve analysis, with phosphorylated tau not included due to the lack of an assay approved for use in patient care in Canada (at present). In this cohort, the A\(\beta\)/A\(\beta\)40 ratio did not improve the AUC relative of A\(\beta\)42 alone. This is compatible with previous studies that have shown similar-to-improved diagnostic performance for A\(\beta\)42/40 versus A\(\beta\)42, compared to amyloid positron imaging tomography scans.14,28 A diagnostic accuracy study, requiring autopsy confirmation, was not undertaken, nor were amyloid positron imaging tomography scans (due to a lack of clinical availability)—diagnostic studies comparing CSF A\(\beta\) to autopsy and/or amyloid imaging tracers has previously been extensively studied for these analytes.29

While the use of mass spectrometry for quantification of peptides presents numerous analytical advantages,13 there is a rarely
In routine care for neurogenerative disorders, an underlying genetic cause can be obscured as family histories may be unknown, uncertain, or miscommunicated to the clinician. Moreover, genetic analysis was not performed as variant identification was deemed sufficient in cases to guide care. For example, identification of a penetrant pathogenic variant is sufficient to identify autosomal dominant AD, and DNA sequencing confirmation would then be recommended. In the case of detection of a non-pathogenic variant, this would prevent reporting of a falsely low Aβ concentration (ie, a concentration based on just the wild-type isoform), and inform appropriate testing (eg, using the location of the sequence variant to select a method likely to detect both isoforms). A limitation of this approach is the identification of new variants, requiring updating of the database and testing as new mutations are identified. For the purposes of demonstrating applicability to a wide range of variants, we included variants based on pathogenicity and a frequency threshold. Given the ease of multiplexing with HPLC-MS/MS, variants monitored can and should be tailored to the laboratory’s clinical population. Thus, this multiplex Aβ HPLC-MS/MS assay serves not only as means to assess the presence of amyloid pathology via quantitation of Aβ peptides, but is also part of the diagnostic workflow for autosomal dominant AD.

On the one hand, Aβ sequence variants only affect accurate quantification of Aβ in a small fraction of individuals (based on reported APP variant frequencies); on the other hand, every case analyzed in clinical care is of equal importance irrespective of variant prevalence. As imprecision and accuracy of Aβ methods have been shown to be important considerations, so is misreporting an Aβ concentration by 50% to 100%, as may occur in cases of APP variant heterozygosity and homozygosity, respectively. Also, there may be yet new roles for variant identification in patient care as genetic research continues to identify new variants and associations with disease, and as mutational databases expand and include more diverse populations. With the availability of a highly selective tool that detects and discriminates Aβ sequence variants, this method can be applied to further our understanding of disease progression and pathology (eg, by studying temporal changes in isoform-specific peptide concentrations).

This is the first method to identify both sporadic AD (by wt-Aβ42 and wt-Aβ40 concentration) and autosomal dominant AD (by identification of pathogenic APP variants) in one method, enabling application of the HPLC-MS/MS method without a priori knowledge of the genetic makeup of an individual. Moreover, Aβ42 has been calibrated to the CRM and the method and the workflow automated on common clinical laboratory equipment. These assay characteristics enabled implementation of the method in routine clinical care.

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**FIGURE 5** ROC curves using the multiplex amyloid β (Aβ) high performance liquid chromatography tandem mass spectrometry assay for: tau/Aβ42 (dark blue), Aβ42 (green), Aβ42/Aβ40 (light blue), and Aβ40 (orange) with total tau measured by enzyme-linked immunosorbertent assay.
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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