Assessment of Peptide Chemical Modifications on the Development of an Accurate and Precise Multiplex Selected Reaction Monitoring Assay for Apolipoprotein E Isoforms

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ABSTRACT: Apolipoprotein E (ApoE) is a polymorphic protein that plays a major role in lipid metabolism in the central nervous system and periphery. It has three common allelic isoforms, ApoE2, ApoE3, and ApoE4, that differ in only one or two amino acids. ApoE isoforms have been associated with the occurrence and progression of several pathological conditions, such as coronary atherosclerosis and Alzheimer’s disease. The aim of this study was to develop a mass spectrometry (MS)-based assay for absolute quantification of ApoE isoforms in cerebrospinal fluid and plasma samples using isotope-labeled peptides. The assay included five tryptic peptides: CLAVYQAGAR (ApoE2), LGADMEDVCGR (ApoE2 and 3), LAVYQAGAR (ApoE3 and 4), LGADMEDVR (ApoE4), and LGPLVEQGR (total ApoE). Both cerebrospinal fluid and plasma samples were assayed to validate the method. The digestion yield and the extension of chemical modifications in selected amino acid residues (methionine oxidation, glutamine deamidation, and cyclization of N-terminus carbamidomethylcysteine) were also studied. The ApoE phenotype was successfully assigned to all samples analyzed in a blinded manner. The method showed good linearity ($R^2 > 0.99$) and reproducibility (within laboratory imprecision <13%). The comparison of the MS-based assay with an ELISA for total ApoE concentration showed a moderate correlation ($R^2 = 0.59$). This MS-based assay can serve as an important tool in clinical studies aiming to elucidate the association between ApoE genotype, total ApoE, and ApoE isoform concentrations in various disorders related to ApoE polymorphisms.

KEYWORDS: apolipoprotein E, cerebrospinal fluid, chemical modification, isoform, peptide, plasma, selected reaction monitoring

INTRODUCTION

Human apolipoprotein E (ApoE) is a 299 amino acid protein with a molecular mass of ~34 kDa, encoded by the APOE gene,
which is localized on chromosome 19. Three major APOE alleles have been identified, named ε2, ε3, and ε4. The APOEε3 is found in more than half of the general population, whereas APOEε4 is present in 5–35% and APOEε2 in 1–5% of individuals. These alleles result in three polymorphic forms of ApoE, which differ by a single amino acid involving cysteine–arginine interchanges: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158), along with six phenotypes: three homozygous (E2/2, E3/3, and E4/4) and three heterozygous (E2/3, E2/4, and E3/4).

ApoE is synthesized by many tissues, with the highest expression in liver and brain. Because this protein does not cross the blood–brain barrier, its secretion and function in blood and cerebrospinal fluid (CSF) are independent. ApoE is one of the key apolipoproteins that regulate the metabolism of lipids by directing their transport, delivery, and distribution in cells and tissues.

ApoE isoforms have been associated with the occurrence or progression of several pathological conditions, such as coronary atherosclerosis, Alzheimer’s disease (AD), and traumatic brain injury. A direct relationship between apoE genotypes and low-density lipoprotein cholesterol (LDL-C) levels and coronary risk is well-established (LDL-C levels and coronary risk, from lower to higher: ε2/ε2 < ε2/ε3 < ε2/ε4 < ε3/ε3 < ε3/ε4 < ε4/ε4). Similarly, the presence of one copy of APOEε4 increases the risk of late-onset AD by three-fold, and the presence of two copies increases the risk by 12-fold. APOEε2 is associated with decreased risk of late-onset AD.

Isoform-specific antibodies against ApoE are scarce, and hence immuno-based assays available for the quantification of ApoE allow the determination of total ApoE concentrations irrespective of ApoE isoform. As with all immuno-based assays, antibody–antigen affinity differences yield varying results between assays where ApoE isoform affinity differences elicit an extra hurdle, possibly masking potential differences in the levels of various ApoE isoforms in heterozygous individuals.

Mass spectrometry (MS)-based methods are becoming an alternative to the enzyme-linked immunosorbent assays (ELISAs) for the analysis and quantification of protein-based biomarkers, like ApoE, with clinical usefulness. Selected reaction monitoring (SRM) assays provide advantages such as high selectivity for protein isoforms, short assay development time, and good multiplexing capabilities. The aim of this study was to develop a MS-based assay for absolute quantification of ApoE isoforms in CSF and plasma samples using isotope-labeled peptides. The digestion yield and the extension of chemical modifications in selected amino acid residues were also studied to ensure an absolute quantification.

## METHODS

### Reagents and Materials

RapiGest SF Surfactant was purchased from Waters Corporation (Milford, MA). Dithiothreitol, iodoacetamide, proteomics-grade trypsin (T6567), trifluoroacetic acid, and phenol were from Sigma-Aldrich (Oakville, ON, Canada). Ammonium bicarbonate (ABC), acetonitrile (grade HPLC) and hydrogen peroxide were from Fisher Scientific (NJ, USA). Formic acid was from EMD (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q purification system (Millipore, Molsheim, France). Four isotopically labeled standard peptides (Spike'Tides TQL) were from JPT Peptide Technologies (Berlin, Germany). One isotopically labeled standard peptide (AQUA peptide LGPLVEQGR) was from Thermo Fisher Scientific (Ulm, Germany). OMIX C18, 10 μL tips were from Agilent Technologies (Lake Forest, CA).

### Sample Collection

CSF samples (n = 20) with known APOE genotype (minisequencing method), ε2/ε3 (n = 5), ε3/ε3 (n = 5), ε3/ε4 (n = 5), and ε4/ε4 (n = 5), were obtained from the Institute of Neuroscience and Physiology, University of Gothenburg (Sweden) and used for initial method development. Additionally, CSF (n = 78) and EDTA-plasma (n = 60) samples with known APOE genotype (but analyzed in a blinded manner) were obtained from the Memory Clinic at Skåne University Hospital in Malmö (Sweden). Five out of six possible phenotypes were present in this second set of samples (except ε2/ε2). An additional set of 10 de-identified CSF samples from the Memory Clinic at Skåne University Hospital in Malmö (Sweden) were used for the comparison between the herein described total ApoE MS method and a conventional sandwich ApoE ELISA. Ethics approval for sample collection was obtained from the respective institutional review boards. All samples were stored at −80 °C until assayed.

### Study of ApoE Isoforms

The four tryptic peptides derived from the two major ApoE single nucleotide polymorphisms (SNP112 and SNP158), 104LGADMEDVCGR114 (from ApoE2&3 and named A), 104LGADMEDVR112 (from ApoE4 and named B), 159LAVY-QAGAR167 (from ApoE3&4 and named C), and 159CLAVQAGAR167 (from ApoE2 and named D), were included in the study as well as one peptide (181LGPLVEQGR189) common to the three isoforms (Figure 1).

For ApoE phenotype identification, the presence of different combinations of these four peptides was used: ε2/ε2 (A+D), ε2/ε3 (A+C+D), ε2/ε4 (A+B+C+D), ε3/ε3 (A+C), ε3/ε4 (A+B+C), and ε4/ε4 (B+C) (Table 1).

![Figure 1. Apolipoprotein E sequence (299 amino acids). The two single nucleotide polymorphisms (SNP112 and SNP158) that generate the three common ApoE isoforms are underlined. The peptide sequences included in the study are highlighted in bold and designated as A–D. See also Table 1](#)
Table 1. Combination of Tryptic Peptides for Each ApoE Phenotype

<table>
<thead>
<tr>
<th>phenotype</th>
<th>isoforms</th>
<th>tryptic peptides</th>
<th>ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>e2/e2</td>
<td>ApoE2</td>
<td>A+D</td>
<td>1:1</td>
</tr>
<tr>
<td>e2/e3</td>
<td>ApoE2 and ApoE3</td>
<td>A+C+D</td>
<td>2:1:1</td>
</tr>
<tr>
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<td>ApoE3</td>
<td>A+C</td>
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</tr>
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<td>A+B+C</td>
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<td>e4/e4</td>
<td>ApoE4</td>
<td>B+C</td>
<td>1:1</td>
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<td>ApoE2 and ApoE4</td>
<td>A+B+C+D</td>
<td>1:1:1:1</td>
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“*Theoretical concentration ratio.

For quantification, three peptides were used: CLAVYQAGAR (ApoE2), LAVYQAGAR (ApoE3), and LGADMEDVR (ApoE4). Because peptide LAVYQAGAR is present in both ApoE3 and 4, the quantification of ApoE3 in samples with phenotype e3/e4 was calculated by subtracting the concentration of LGADMEDVR (ApoE4) from LAVYQAGAR (ApoE3 and 4). Peptide LGPLVEQGR was used only for ApoE phenotype identification. Peptide LGPLVEQGR (total ApoE) was used to study the accuracy of ApoE isoform concentration by comparing the total ApoE concentration calculated from the sum of the different ApoE isoforms with the total ApoE concentration obtained from this peptide.

Several chemical modifications in these peptides were also studied including methionine oxidation, glutamine deamidation, and cyclization of N-terminus carbamidomethylcysteine. In total, 28 tryptic peptides from ApoE were analyzed (Table S1 in the Supporting Information). One peptide from Apolipoprotein B (ApoB), a plasma-derived protein that is absent in the central nervous system (CNS), was assayed to check for blood contamination in CSF samples. Peptide uniqueness was confirmed by searching against the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/).

Peptide Identification

Peptides were initially identified in an LTQ Orbitrap XL (Thermo Fisher, San Jose, CA) by analyzing a pool of CSF samples. The pool was processed as previously described. The resulting spectra were searched separately against the International Protein Index human database v3.71 (modified by adding the FASTA sequences of ApoE isoforms 2 and 4) by Mascot software (version 2.2, Matrix Science). The following parameters were used: (i) enzyme, trypsin; (ii) one missed cleavage allowed; (iii) fixed modification, carbamidomethylation of cysteines; (iv) variable modifications, oxidation and deamidation of methionines, deamidation of glutamines, and pyro-carbamidomethylation of N-terminus cysteines; (v) peptide tolerance, 7 ppm; and (vi) MS/MS tolerance, 0.4 Da. The resulting Mascot search files were uploaded into Scaffold, version 2 (Proteome Software).

Sample Preparation for SRM Analysis

Before analysis, samples were thawed at room temperature and centrifuged at 17 000 g for 10 min. Twenty-five microliters of CSF and 15 μL of plasma (previously diluted 1:100 with 50 mM ABC), which is equivalent to 8–12 μg of total protein, were denatured and reduced with 0.05% RapiGest and 5 mM dithiothreitol for 40 min at 60 °C. Alkylation was performed using 15 mM iodoacetamide for 60 min at room temperature and in the dark. More dithiothreitol was added to a 10 mM final concentration (to quench excess iodoacetamide), and the samples were incubated for 15 min at room temperature. A mixture of four isotopically labeled peptides (SpikeTides TQL) was added (100 and 25 fmol of each peptide (per run) for CSF and plasma samples, respectively). The proteins/standard peptides were digested with trypsin for 24 h at 37 °C (trypsin to total protein ratio 1:30 and 1:10 (w/w) for CSF and plasma samples, respectively). After trypsinization, the AQUA standard peptide was added (same concentrations as SpikeTides peptide) and RapiGest was cleaved with 1% trifluoroacetic acid. The samples were centrifuged at 500g for 30 min for RapiGest precipitation. Peptides were purified and extracted using OMIX C18 tips and then eluted using 5 μL of 65% acetonitrile solution (0.1% formic acid). Finally, the peptides were diluted with 60 μL of water (0.1% formic acid). All steps were performed in a 96-well plate.

Liquid Chromatography–Mass Spectrometry (LC–MS) Conditions

Samples were loaded onto a 2 cm trap column (C18, 5 μm) with an inner diameter of 150 μm, and the peptides were eluted onto a resolving column analytical column (TSQ Vantage, Thermo Fisher, San Jose, CA) using a nanoelectrospray ionization source (nano-ESI, Thermo Fisher). A three-step 30 min gradient with an injection volume of 18 μL was used. Buffer A contained 0.1% formic acid in water, and buffer B contained 0.1% formic acid in acetonitrile. Peptides were analyzed by SRM assays with the following parameters: positive-ion mode, predicted collision energy values, optimized scan times, 0.2 Th of full width at half-maximum (fwhm) in Q1 and 0.7 Th in Q3, 1.5 mTorr Q2 pressure, tuned tube lens values, and 1 V skimmer offset. In-silico digestion, fragmentation, and prediction of collision energy were performed using Skyline software, version 1.4.12

Selection of Transitions

Initially, all transitions (from y1 to yn) were monitored in a pool of CSF samples. Then, three transitions for each endogenous and isotope-labeled peptide were selected based on two main criteria: relative intensity of transitions and presence of interferences. Transitions with the highest intensity were preferred. The presence of interferences was predicted by using the SRM collagen software, version 1.4 (www.srmcollider.org). This software predicts unique ion signatures (UISs) for each peptide. The search parameters were: SSRCalc window: 10 arbitrary units; Q1 mass window: 0.7 Th; low and high mass threshold for transitions: 300 and 1500 Th, respectively; genome: Human Peptide Atlas; consider isotopes up to 3 amu; one missed cleavage; find UIS up to order 3; and finally, charge check, modifications, and all background ion series were selected.

Digestion Optimization

To analyze the efficiency of trypsin digestion for peptides LGADMEDVCGR, LGADMEDVR, LAVYQAGAR and CLAVYQAGAR, we processed 16 aliquots (10 μg of total protein each) of a pool of CSF samples as previously described. Before digestion, the aliquots were mixed and homogenized to avoid any variability between samples and divided again into 16 parts for trypsin digestion. Samples were digested at 37 °C (trypsin to total protein ratio 1:30 (w/w)) for 9, 12, 15, 18, 21, and 24 h (two or three aliquots at each time point). The samples were analyzed in triplicate in a random order. The ratio
(light/heavy) for each peptide was compared with the ratio at 24 h (final time point) and displayed as percentage ("relative digestion efficiency").

In a second digestion study, two pools of CSF (6 aliquots of 25 μL) and plasma (6 aliquots of 15 μL) samples were analyzed in duplicate. The efficiency of trypsin digestion for peptides LGADMEDVCGR, LGADMEDVR, LAVYQAGAR, CLAVYQAGAR, and LGPLVEQGR was evaluated. The trypsin to total protein ratio was set to 1:30 (CSF) and 1:10 (plasma), respectively, and the samples were digested at 37 °C for 5, 12, 15, 18, 21, and 24 h (one aliquot at each time point). Times longer than 24 h were not tested.

**Study of Chemical Modifications**

**Oxidation of Methionines.** Two CSF samples (with ApoE phenotype ε3/ε3 and ε4/ε4) were treated with different concentrations of performic acid/phenol after following the previously described protocol in the manuscript (see Sample Preparation for SRM Analysis section) and before peptide extraction with C18 tips. The solution was freshly prepared by mixing 900 μL of formic acid with 5 mg of phenol. Then, it was cooled at 0 °C and 100 μL of 30% hydrogen peroxide was added. The resulting solution was stirred gently at room temperature for 30 min. Finally, the performic acid solution was diluted 1:10 and 1:100. Eight CSF aliquots (4 aliquots of ApoE3 and 4 aliquots of ApoE4 samples) were treated with 5 μL of performic acid solutions (nondiluted, diluted 1:10 and diluted 1:100) or 5 μL of 50 mM ABC. The aliquots were incubated for 24 h at 4 °C and analyzed, after peptide extraction with C18 tips, with the SRM method described in Table S2 in the Supporting Information.

**Deamidation of Glutamines to Glutamic Acid.** Initially, an experiment to verify whether the resolution used in our SRM assay was appropriate to independently detect peptides with nondeamidated and deamidated glutamines (LAVYQAGAR, CLAVYQAGAR, and LGPLVEQGR) was performed. A pool of plasma samples was analyzed five times with a method (Table S3 in the Supporting Information), in which different resolutions in Q1 were used (fwhm: 0.06, 0.1, 0.2, 0.4, and 0.7 Th). Subsequently, two pools of CSF and plasma samples as well as three individual plasma samples (with ApoE phenotype ε2/ε3 and ε2/ε4) were analyzed (fwhm (Q1): 0.2 Th). The extension of glutamine deamidation across peptides and the impact of this modification in the calculation of absolute concentrations were calculated. The differences in the estimation of peptide concentration when all transitions (from y₁ to y₀₋₁) or only three transitions were used (in both nondeamidated and deamidated forms) were also studied in the CSF pool.

**Cyclization of N-terminus Cysteines.** Both noncycled and cyclized forms of peptide CLAVYQAGAR were performed in the analysis of CSF and plasma samples.

**Quantification of ApoE Isoforms in CSF and Plasma Samples**

The samples were analyzed using the SRM method described in Table S4 in the Supporting Information.

**Linearity of the Assay.** Calibration curves with different concentrations (1000, 100, 10, and 1 fmole on column) of isotopically labeled peptides CLAVYQAGAR, LAVYQAGAR, LGADMEDVR, and LGPLVEQGR and constant concentration of endogenous peptides (CSF pool) were prepared and assayed. Calibration curves for ApoE4 were also prepared and assayed just before the samples to normalize the digestion yield of endogenous peptide LGADMEDVR and the extension of methionine oxidation in the isotope-labeled peptide. Two pools of CSF and plasma samples with phenotype ε4/ε4 were prepared and diluted (1:2) four consecutive times with samples with ApoE phenotype ε3/ε3 (and similar total protein concentration). The ApoE4 concentration in the initial samples was calculated with the peptide LAVYQAGAR.

**Reproducibility.** Four “quality controls” (two CSF and two plasma samples) were included in the analysis. These quality controls were prepared by mixing two samples with APOE2/4 and 3/3 (named QC1) and two samples with APOE2/3 and 4/4 (named QC2). Four aliquots of each quality control were processed simultaneously with the samples and analyzed over several days. The results (16 CSF and 16 plasma values) were analyzed following the recommendations of Clinical and Laboratory Standards Institute (CLSI), document EP5-A2.14

**Correlation of Total ApoE Concentrations between SRM and ELISA**

In the additional set of 10 CSF samples used for method comparisons, the total ApoE concentrations were assayed with an in-house developed sandwich ELISA at the Mayo Clinic and then sent to Toronto for MS analysis. The sandwich ELISA was developed using a well-characterized monoclonal anti-ApoE antibody (WUE4),15 previously employed for similar purposes,16 as capture antibody and a biotinylated goat anti-ApoE detection antibody (Meridian Life Science). A 96-well plate (NUNC MaxiSorb) was coated with 1 μg/mL capture antibody in carbonate buffer pH 9.6 overnight at room temperature (RT). Following washing with PBS-T (0.05% Tween20 in PBS), the plate was blocked with 1% milk in PBS for a minimum of 1h at RT. Recombinant ApoE (Fitzgerald Industries International), concentration range 250–16000 pg/mL, and CSF samples were diluted in blocking buffer and added in duplicate to the wells. Standards and samples were incubated for 2 h at RT. The plate was washed and 100 ng/mL detection antibody in blocking buffer was applied to each well, followed by another 2 h of incubation at RT. Streptavidin poly-HRP40 (Fitzgerald) 1:1000 in blocking buffer was added after another wash step, and the plate was incubated at RT for 30 min and again washed. The peroxidase substrate TMB Super Slow (Sigma-Aldrich) was added to the wells, and after 10 min of incubation at RT in the dark the colorimetric reaction was stopped with 1 M sulfuric acid. The optical density at 540 nm was quantified, and duplicate readings of standards and samples were averaged. The ApoE concentrations were determined by interpolation from a four-parametric fitted calibration curve. The intra- and interassay coefficients of variation (CV) were lower than 10 and 15%, respectively.

**Data Analysis**

All samples, quality controls, and calibration curves were randomized and analyzed in duplicate. The raw files were uploaded to Pinpoint software, version 1.0 (Thermo Fisher), which was used for quantification of areas under the curve. CVs and Pearson product-moment correlation were calculated with IBM SPSS Statistic software, version 20.
RESULTS

Peptide Identification

ApoE peptides were initially searched and identified in a pool of CSF samples analyzed by LC–MS/MS by using Scaffold software. Peptides with mono-oxidation (LGADM[+16]EDVCGR and LGADM[+16]EDVR) but not dioxidation of methionines were found. The peptide with a pyro-carbamido-methyl cysteine residue (C[+40]LAVYQAGAR) was also identified. Glutamine deamidation was found only in the peptide LGPLVEQ[+1]GR (common to the three ApoE isoforms).

For confirmation of peptide identity, the relative intensity of the transitions (from \( y_3 \) to \( y_{n-1} \)) obtained in the TSQ Vantage were compared with the intensity of transitions from discovery data (LTQ Orbitrap XL). The MS spectra and identification of peptides are displayed in Figures S1–S10 in the Supporting Information.

Digestion Optimization

The initial analysis of a pool of CSF samples showed that while the digestion (ratio 1:30, trypsin:total protein) of peptides LAVYQAGAR and CLAVYQAGAR seemed to be complete, with the concentration of these two endogenous peptides progressing linearly from 9 to 24 h of incubation (Figure S11 in the Supporting Information). These results were independently verified in a second analysis (Figure S12A in the Supporting Information), where the digestion of peptide LGPLVEQGR was also included.

When the ratio trypsin/total protein was increased from 1:30 to 1:10 (plasma samples), the digestion of four out of five peptides was clearly completed after 15–18 h of incubation (Figure S12B in the Supporting Information). The digestion of peptide LGADMEDVR was the most difficult, but complete digestion was achieved after 21 h of incubation (Figure S12B in the Supporting Information).

To further check the efficiency of trypsin digestion of peptide LGADMEDVR, the ratios (light/heavy) obtained for peptides LAVYQAGAR and LGADMEDVR in samples with ApoE phenotype \( \varepsilon4/\varepsilon4 \) were compared. In these samples, the concentration of both peptides should be equal because both peptides come from the same isoform (Table 1). The analysis of CSF samples (\( n = 8 \)) revealed that the digestion yield was, on average, 7% (trypsin to total protein ratio 1:30), while the yield in plasma samples (\( n = 6 \)) was 81% (trypsin to total protein ratio 1:10).

Study of Chemical Modifications

Oxidation of Methionines. This study was performed to identify the peptides with mono- and dioxidized methionines and analyze whether the dioxidation of peptides containing methionine residues could be a reliable method for quantitative analysis of ApoE, as previously described.17

After sample oxidation, the mono- (5.7 min) and dioxidized (6.0 min) forms of peptide LGADMEDVR were found to be more hydrophilic that the nonoxidized form (8.1 min.). The mono-oxidized peptide LGADMEDVCGGR also eluted at a shorter retention time (6.2 min.) than the nonoxidized form (8.2 min.) (Supplemental data, Figure S13).

The oxidation reaction resulted in the complete conversion of peptide LGADMEDVR to the dioxidized form LGADM-[+32]EDVR (in a CSF sample with ApoE4/4) when the most concentrated oxidizing solution was used (Figure S14 in the Supporting Information). However, solutions diluted 1:100 and 1:10 were not as effective, and only a partial mono-oxidation of methionine was observed (LGADM[+16]EDVR). Other peptides from ApoE isoforms included in the analysis were also affected by this oxidation reaction. Thus, the concentration of peptide LGADMEDVCGGR (in a CSF sample with ApoE 3/3) was reduced in the aliquots treated with oxidizing solution diluted 1:100 and 1:10 (formation of mono-oxidized form) and was not detectable in the aliquot treated with the highest concentration of performic acid. However the dioxidized peptide LGADM[+32]EDVCR was not detected. The amount of peptides LAVYQAGAR and CLAVYQAGAR (using the most concentrated oxidizing solution) was <5% of the concentration in the sample treated with 50 mM ABC. The concentration of peptide LGPLVEQGR was not significantly affected by this reaction.

When sets of CSF and plasma samples were assayed, the degree of oxidation in methionine residues was estimated by calculating the ratio between the mono-oxidized and non-oxidized forms of peptides LGADMEDVR and LGADMEDVCGGR.

The percentage of oxidation (average and [minimum–maximum]) of peptide LGADMEDVR (in 37 CSF and 30 plasma samples with ApoE4, respectively) was: 1% [0–2%] and 2% [1–5%] (endogenous peptide); 4% [3–7%] and 4% [3–6%] (isotope-labeled peptide).

The percentage of oxidation (average and [minimum–maximum]) of peptide LGADMEDVCGGR (in 70 CSF and 54 plasma samples with ApoE2 and/or ApoE3) was: 2% [0–5%] and 3% [1–7%] (endogenous peptide); 10% [5–20%] and 18% [13–29%] (isotope-labeled peptide).

Deamidation of Glutamines. The goal of this experiment was to evaluate the impact of glutamine deamidation in the calculation of peptide concentration.

The nondeamidated and deamidated forms of peptides LAVYQAGAR, CLAVYQAGAR, and LGPLVEQGR showed the same retention times (Figure S15 in the Supporting Information). The percentage of glutamine deamidation obtained for the different peptides when all transitions (from \( y_3 \) to \( y_{n-1} \)) or only three transitions were used for quantification was very similar (<3% variation, data not shown). The resolution used in our SRM assay (0.2 Th in Q1 and 0.7 Th in Q3) was appropriate to independently identify both deamidated and nondeamidated forms of the peptides under study. There was no variation in the light/heavy ratios when resolution in Q1 was changed from 0.06 to 0.7 Th (data not shown), probably because the product ions are well-resolved in Q3 (difference of 1 Da between nondeamidated and deamidated forms).

The extension of glutamine deamidation for each peptide across samples was very reproducible, with a variation not higher than 3%. This deamidation was slightly higher in the endogenous peptides than in the isotope-labeled peptides. Thus, the conversion of endogenous peptides was from 29 to 46%, whereas the conversion of isotope-labeled peptides was from 22 to 37% over a 24 h period (Figure S15D in the Supporting Information).

When the intensities of both nondeamidated and deamidated forms of each peptide were added for calculation of peptide concentration and compared with the concentration obtained using only the nondeamidated form, it was found that there was

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a difference in concentration of 5 to 6% (Table S5 in the Supporting Information).

**Cyclization of N-Terminus Carboxamidomethylcysteines.** The cyclization of N-terminus cysteines during trypsin digestion has been previously described. The noncyclized (9.3 min) and cyclized (15.0 min) forms of peptide CLAVYQAGAR showed quite different retention times (Figure S16 in the Supporting Information). The quality control report of isotope-labeled peptide, provided by JPT Company, showed a partial cyclization of this peptide during the synthesis process (Figure S17 in the Supporting Information). The quantification of both forms with the JPT tag was used to estimate the percentage of cyclized (28%) and noncyclized (72%) peptides for subsequent calculations.

The extension of cyclization in peptide CLAVYQAGAR (in sets of CSF and plasma samples) was estimated by calculating the ratio: cyclized/(noncyclized+cyclized) peptide forms. The percentage of cyclization (average and [minimum–maximum]) in 10 CSF and 6 plasma samples with ApoE2 was, respectively: 51% [34–63%] and 60% [51–69%] (endogenous peptide); 71% [58–78%] and 74% [67–81%] (isotope-labeled peptide). This reaction progressed in a very similar manner across different samples (Figure S18 in the Supporting Information); however, the degree of cyclization in endogenous and isotope-labeled peptides in CSF and plasma samples was different.

To check the reproducibility of this reaction, we repeated two CSF samples (with ApoE phenotype ε2/ε3 and ε2/ε4) 2 weeks after the first analysis, following the same protocol but reducing the volume from 25 to 15 μL of CSF (limited availability of sample). The concentration of ApoE2 was calculated, being 2 and 7% lower, respectively, in the second analysis.

**ApoE Phenotype Identification**

The ApoE phenotype was established by using the combinations of peptides described in the Methods section. Depending on the presence or absence of these peptides, the ApoE phenotype was assigned (Figure 2 and Suppilmental data, Figures S19 to S22). This phenotype was successfully assigned to all samples analyzed in a blinded manner (n = 138) when compared with genetic testing results (100% success rate).

**Quantification of ApoE Isoforms**

**Linearity of the Assay.** Peptides CLAVYQAGAR, LAVYQAGAR, LGADMEDVR, and LGPLVEQGR showed good linearity between 1 and 1000 fmol (on column), with good coefficients of determination, R² > 0.99 (Figure 3).

The correlation between the total ApoE concentration calculated by adding the concentration of each isoform (peptides CLAVYQAGAR, LAVYQAGAR, and LGADMEDVR) or obtained directly from the concentration of peptide LGPLVEQGR (Figure 4) showed similar correlation (R = 0.98) but somewhat different results in CSF and plasma samples. Thus, the concentration of total ApoE calculated was higher (19% on average) across all CSF samples (except one), while it was slightly lower (15% on average) across all plasma samples. Similar results were obtained with the QCs. The two CSF-QCs showed a 13% higher concentration of total calculated ApoE, whereas the two plasma controls showed a 15% (QC1) and 18% (QC2) lower concentration (Table S6 in the Supporting Information).

Furthermore, the degree of cyclization of endogenous peptide CLAVYQAGAR was higher in plasma samples than in CSF samples, which could lead to an underestimation of the absolute concentration of ApoE2. It was observed that the plasma samples with this isoform showed lower values of total calculated ApoE (Figure S24 in the Supporting Information).

**Correlation between Total ApoE Concentrations.**

The comparison of total ApoE concentration calculated by adding the concentration of each isoform (peptides CLAVYQAGAR, LAVYQAGAR, and LGADMEDVR) or obtained directly from the concentration of peptide LGPLVEQGR (Figure 4) showed similar correlation (R = 0.98) but somewhat different results in CSF and plasma samples. Thus, the concentration of total ApoE calculated was higher (19% on average) across all CSF samples (except one), while it was slightly lower (15% on average) across all plasma samples. Similar results were obtained with the QCs. The two CSF-QCs showed a 13% higher concentration of total calculated ApoE, whereas the two plasma controls showed a 15% (QC1) and 18% (QC2) lower concentration (Table S6 in the Supporting Information).

**Correlation of Total ApoE Concentration between SRM and ELISA**

Four ApoE phenotypes were identified in the 10 CSF samples assayed: ε2/ε3 (n = 1), ε2/ε4 (n = 1), ε3/ε3 (n = 3) and ε3/ε4 (n = 5). The correlation between the total ApoE concentration calculated by adding the concentration of each isoform or obtained directly from the concentration of peptide
LGPLVEQGR was very high ($R^2 > 0.99$) (Figure 5A), while the correlation between the SRM assay and the ELISA was moderate ($R^2 = 0.59$) (Figure 5B).

An optimized workflow of the proposed SRM assay for quantification of ApoE isoforms is displayed in Figure 6.

## DISCUSSION

MS has emerged as a technology that offers novel applications in clinical laboratories and can solve some of the limitations of immunoassays. SRM methods require shorter times for assay development, are cost-effective due to their superior multiplexing capabilities, and provide the required sequence specificity to discriminate and measure clinically relevant protein isoforms.19-23

Wildsmith et al.24 were the first to show the feasibility of identifying and quantifying (relatively) ApoE isoforms in CSF and astrocyte culture media, by using an MS-based method. Later, the same group used this technology to study the ApoE turnover rates in the CNS.25 However, this approach is limited
to comparative measurements in cell cultures. Simon et al.\textsuperscript{17} published an SRM method for absolute quantification of ApoE4 and total ApoE in plasma by analyzing the peptides LGADMEDVR and LGPLVEQGR after oxidation of methionine residues. Very recently, Krastins et al.\textsuperscript{26} developed SRM assays for quantification of total ApoE and ApoE isoforms after immunoenrichment of plasma samples.

We evaluated an MS-based method for simultaneous identification and absolute quantification in CSF and plasma samples of ApoE isoforms and total ApoE by using five isotope-labeled peptides. The digestion yield and the extension of chemical modifications in some amino acid residues were also studied.

Trypsin digestion is a crucial step to provide an absolute quantification of proteins.\textsuperscript{27} There are no optimal digestion conditions because this process is trypsin- and protein-dependent.\textsuperscript{29} We initially evaluated the digestion yield in CSF samples, where important differences between peptides were found (Figure S11 in the Supporting Information). These results were confirmed in a second analysis (Figure S12A in the Supporting Information). To enhance the digestion efficiency in plasma samples, we increased the amount of trypsin from 1:30 to 1:10. This modification improved the digestion yield because the digestion of all peptides was complete after 24 h of incubation (Figure S12B in the Supporting Information). However, the digestion of peptide LGADMEDVR continued to be the most challenging. These differences between peptides could be associated with the sequence and structure of ApoE isoforms. It has been described that the presence of Arg112 in ApoE4 affects the conformation of the side chain of Arg61, which produces the interaction between Arg61 and Glu255\textsuperscript{30} and could affect the digestion of peptide LGADMEDVR. The apparent small reduction in peptide concentration between 21 and 24 h of incubation, if real, might be associated with an inconsistency in trypsin digestion, as it was previously described for other proteins.\textsuperscript{29} In the case of ApoE, only Proc et al.\textsuperscript{29} previously studied the trypsin digestion of this protein, but that study was limited to peptide LGPLVEQGR.

Chemical modifications, both in vivo and in vitro, occurring in peptide sequences due to sample processing have to be considered because the targeted peptides might be converted into the modified form in an irreproducible and unpredictable manner.\textsuperscript{31} The most commonly described chemical modifications include methionine and tryptophan oxidation, asparagine and glutamine deamidation, and cyclization of N-terminus glutamines.\textsuperscript{31,32} In the present study, we examined the oxidation of methionine residues, glutamine deamidation, and the N-terminus cyclization of carbamidomethylcysteines.

First, we performed an oxidation experiment for methionine residues during which two samples were incubated with different concentrations of an oxidizing solution (performic acid/phenol). The retention times obtained for peptide LGADMEDVR, 5.7 (mono-oxidized), 6.0 (dioxidized) and 8.1 min (nonoxidized), were very similar to the retention times reported by Simon et al.\textsuperscript{17} (4.2, 6.5, and 8.1 min, respectively). The oxidation of methionine (M) to methionine sulfone (M[+32]) is an irreversible reaction\textsuperscript{33} that can be used to accurately quantify peptides with methionine residues.\textsuperscript{17} In this experiment, the oxidation of peptide LGADMEDVR was complete; however, other peptides were also chemically modified in the process. The peptide LGADM[+32]EDVCGR was not detected in any of the aliquots analyzed, probably due to the oxidation of cysteine residues. Peptides LAVYQAGAR and CLAVYQAGAR were still detectable when the most concentrated oxidizing solution (performic acid/phenol) was used, but their relative intensity decreased to <5% of original signal (Figure S14 in the Supporting Information). This was probably due to the oxidation of cysteines and halogenation of tyrosines.\textsuperscript{34,35} These side reactions could make the analysis of these peptides irreproducible, and thus we decided to exclude this reaction from subsequent analyses.

The natural oxidation of methionine to sulfoxide methionine was estimated in all CSF and plasma samples. The degree of oxidation of endogenous peptides LGADMEDVR and LGADMEDVCGR was very low. However, the oxidation in isotope-labeled peptide LGADMEDVR was slightly higher, and the isotope-labeled peptide LGADMEDVCGR showed an
increased oxidation in CSF and plasma samples. It is important to point out that different vials for all isotope-labeled peptides were utilized for the analysis of CSF (analyzed first) and plasma samples (analyzed later). These results highlight the instability of peptide LGADMEDVCGGR and its propensity to oxidation (under storage conditions), probably due to the presence of methionine and cysteine residues in the sequence. Therefore, this peptide was not considered appropriate for quantification purposes because the higher degree of oxidation in isotope-labeled peptide results in an overestimation of endogenous peptide concentration.

The conversion of glutamine residues to glutamic acid was also studied. Both nondeamidated and glutamine deamidated peptides showed identical retention times. Glutamine deamidated peptides are slightly more hydrophobic than the nondeamidated forms, but they can only be chromatographically separated by using very long gradients. The extent of this reaction varied across peptides because the conversion rate of glutamine to glutamic acid is dependent on the surrounding amino acid sequence. Moreover, the conversion rate in endogenous peptides was found to be slightly higher than the conversion rate in isotope-labeled peptides (Figure S15D in the Supporting Information). The deamidation of glutamine and asparagine residues in proteins has been extensively studied, and it has been hypothesized that the conversion of these residues serves as a molecular timer of biological events such as protein turnover, development, and aging. This could explain the higher degree of deamidation observed in the endogenous peptides, which produced an underestimation in the concentration of 5 to 6%. The detection of both forms was not included in the analysis of CSF and plasma samples because the underestimation in absolute concentration was considered not relevant. However, an accurate calculation of absolute concentrations of peptides containing glutamine or asparagine residues requires the analysis of both forms.

The cyclization of N-terminus carbamidomethylcysteines was previously studied. Reimer et al. described increased retention times in peptides with N-terminus cyclization, and we also found that the cyclized peptide C+[40]LAVYQAGAR is more hydrophobic than the noncyclized form (15.0 min versus 9.3 min). Krokhin et al. showed that the degree of peptide conversion depends on digestion time, with an average cyclization of 51%, after a typical overnight digestion at 37 °C. We obtained a similar degree of conversion (55% on average). According to our results, this reaction seems to be very reproducible when the same conditions are used, allowing the utilization of peptides with this modification for relative quantification. For absolute quantification, we needed to recalculate the concentrations because the isotope-labeled peptide was already partially cyclized and assumed that the degree of cyclization in endogenous and isotope-labeled peptides (during trypsin digestion) would be the same. However, this assumption may not be accurate because the degree of conversion of endogenous peptide in plasma was higher than that in CSF samples (Figure S18 in the Supporting Information) and the absolute quantification of the peptide seemed to be underestimated (by ~20%).

The reproducibility and linearity of the multiplex SRM assays were also studied. The within-laboratory precision was lower than 13% for all peptides, and the analytical range evaluated showed good linearity with $R^2 > 0.99$.

The comparison of total ApoE concentrations obtained with the sum of ApoE isoforms and with peptide LGPLVEQGR showed good correlation ($R = 0.98$) in both CSF and plasma samples, with a difference of about ±20% between both methods. The lower total ApoE concentrations with peptide LGPLVEQGR in CSF samples were probably due to an incomplete digestion of this peptide.

Finally, despite optimizing a previously described ApoE ELISA employing the well-characterized anti-ApoE antibody WUE4 and despite excellent assay performance data, the correlation between the SRM assay and the ELISA was moderate ($R^2 = 0.59$) (Figure S5B). This is in line with data from a previously reported comparison between total ApoE concentrations determined using a WUE4-based ApoE ELISA and total ApoE concentrations as quantified using MS. Various ELISAs for total ApoE quantification have been previously used and described, showing significantly different results and highlighting a lack of reliability and, therefore, the need for a reference method.

The proposed SRM showed good performance for identification and quantification of ApoE isoforms. This assay can be used to elucidate the isoform composition of total ApoE in CSF and plasma samples from patients with neurodegenerative diseases (such as AD) and nondemented controls.

However, several limitations need to be considered. For clinical applications involving detection of biomarker proteins, the digestion protocols should be optimized for the target proteins of interest. Moreover, chemical modifications in target peptides need to be evaluated, as these may affect accuracy of measurement of peptide concentration. Our results were not significantly affected by the oxidation of methionines or the deamidation of glutamines. However, the cyclization of N-terminus cysteines affected the accuracy of results in plasma samples. To avoid the previously described shortcomings, we could solve this limitation using optimization of digestion conditions, the use of calibration curves (with $ε/ε_2$ samples), or the design of concatamer standards (QconCAT) with this peptide in an internal position of the sequence. Another possibility is the use of Protein Standard Absolute Quantification (PSAQ) as it has been described. However, this approach may substantially increase the assay development times and the cost of analysis.

## Conclusions

We here describe a novel multiplex SRM assay for simultaneous quantification of total ApoE as well as the absolute concentrations of the various ApoE isoforms in APOE homozygous and heterozygous individuals. This method can serve as a significant tool in clinical studies aiming to elucidate the association between APOE genotype, total ApoE, and ApoE isoform concentrations in various disorders related to APOE polymorphism.

## Associated Content

### Supporting Information

Proteotypic peptides included in the study. SRM parameters used for each proteotypic peptide in the oxidation and deamidation studies and in the analysis of CSF and plasma samples. Variation in the concentration of peptides LAVYQAGAR, C+[57]LAVYQAGAR, C+[40]LAVYQAGAR, and LGPLVEQGR, in five samples due to glutamine deamidation.
(CV_{ntr}a, CV_{ntr}b, and CV_{total}) obtained in two levels of control (QC1 and QC2) prepared from two pools of CSF and plasma samples. MS/MS spectrum of the doubly charged peptides LGADMEDVC[+57]+GR (m/z 611), LGADM[+16]EDVC[+57]+GR (m/z 619), LGADMEDVR (m/z 503), LGADM[+16]EDVR (m/z 511), LAVYQAGAR (m/z 474), C[+57]-LAVYQAGAR (m/z 554), C[+40]-LAVYQAGAR (m/z 546), LGPLVEQGR (m/z 484), LGPLVEQ[+1]GR (m/z 485), and GFEPTLEALFGK (m/z 654) acquired on an LTQ Orbitrap XL. Digestion of four peptides used in the analysis of ApoE isoforms at six time points. Digestion efficiency for trypsin to total protein ratios of 1:30 and 1:10 in a pool of CSF and plasma samples, respectively. Elution of mono-oxidized, di-oxidized, and non-oxidized forms of endogenous and isotope-labelled standard peptides LGADMEDVR. Oxidation of peptide LGADMEDVR (in CSF sample with ApoE4/4) to the di-oxidized form LGADM[+32]+EDVR with performic acid/phenol. Co-elution of three transitions (y-ions) of each endogenous and isotope-labelled standard (glutamine-deamidated and non-deamidated) peptides LAVYQAGAR, CLAVYQAGAR, and LGPLVEQGR. Retention time of endogenous and isotope-labelled standard peptides C[+57]-LAVYQAGAR (9.3 min) and C[+40]-LAVYQAGAR (15.0 min) in a 30 min gradient. Quantification of the isotope-labelled standard peptide CLAVYQAGAR after synthesis. Mathematical formula used to estimate the degree of cyclization in endogenous and isotope-labelled peptide CLAVYQAGAR. Identification of endogenous peptides in CSF sample from individual with ApoE phenotype ε2/ε3, ε3/ε3, ε3/ε4, and ε4/ε4. Calibration curves obtained from serial dilutions of two pools of CSF and plasma samples with ApoE phenotype ε4/ε4. Improvement in the correlation between total ApoE calculated and total ApoE obtained from peptide LGPLVEQGR by increasing by 20% the concentration of ApoE2. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS:**

ABC, ammonium bicarbonate; AD, Alzheimer’s disease; ApoB, apolipoprotein B; ApoE, apolipoprotein E; CLSI, Clinical and Laboratory Standards Institute; CNS, central nervous system; CSF, cerebrospinal fluid; R², coefficient of determination; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; fwhm, full width at half-maximum; LC, liquid chromatography; LDL-C, low-density lipoprotein cholesterol; MS, mass spectrometry; QC, quality control; RT, room temperature; SNP, single nucleotide polymorphism; SRM, selected reaction monitoring; UIS, unique ion signature.

**REFERENCES**


