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Romain Simon, Marion Girod, Catherine Fonbonne, Arnaud Salvador, Yohann Clément, et al.. Total ApoE and ApoE4 isoform assays in an alzheimer's disease case-control study by targeted mass spectrometry (n=669): a pilot assay for methionine-containing proteotypic peptides. *Molecular and Cellular Proteomics*, 2012, 11 (11), pp.1389-1403. 10.1074/mcp.M112.018861 . hal-00875286

HAL Id: hal-00875286

<https://hal.science/hal-00875286>

Submitted on 4 Sep 2020

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Total ApoE and ApoE4 Isoform Assays in an Alzheimer's Disease Case-control Study by Targeted Mass Spectrometry ($n = 669$): A Pilot Assay for Methionine-containing Proteotypic Peptides*[§]

Romain Simon‡, Marion Girod‡, Catherine Fonbonne‡, Arnaud Salvador‡, Yohann Clément‡, Pierre Lantéri‡, Philippe Amouyel§¶, Jean Charles Lambert§¶||, and Jérôme Lemoine‡||

Allelic polymorphism of the apolipoprotein E (ApoE) gene (ApoE ϵ 2, ApoE ϵ 3 and ApoE ϵ 4 alleles) gives rise to three protein isoforms (ApoE2, ApoE3 and ApoE4) that differ by 1 or 2 amino acids. Inheritance of the ApoE ϵ 4 allele is a risk factor for developing Alzheimer's disease (AD). The potential diagnostic value of ApoE protein levels in biological fluids (*i.e.* cerebrospinal fluid, plasma and serum) for distinguishing between AD patients and healthy elderly subjects is subject to great controversy. Although a recent study reported subnormal total ApoE and ApoE4 levels in the plasma of AD patients, other studies have found normal or even elevated protein levels (*versus* controls). Because all previously reported assays were based on immunoenzymatic techniques, we decided to develop an orthogonal assay based on targeted mass spectrometry by tracking (i) a proteotypic peptide common to all ApoE isoforms and (ii) a peptide that is specific for the ϵ 4 allele. After trypsin digestion, the ApoE4-specific peptide contains an oxidation-prone methionine residue. The endogenous methionine oxidation level was evaluated in a small cohort ($n = 68$) of heterozygous ϵ 3 ϵ 4 carriers containing both healthy controls and AD patients. As expected, the proportion of oxidized residues varied from 0 to 10%, with an average of 5%. We therefore developed a standardized strategy for the unbiased, absolute quantification of ApoE4, based on performic acid oxidation of methionine. Once the sample workflow had been thoroughly validated, it was applied to the concomitant quantification of total ApoE and ApoE4 isoform in a large case-control study ($n = 669$). The final measurements were consistent with most previously reported ApoE concentration values and confirm the influence of the different

alleles on the protein expression level. Our results illustrate (i) the reliability of selected reaction monitoring-based assays and (ii) the value of the oxidation step for unbiased monitoring of methionine-containing proteotypic peptides. Furthermore, a statistical analysis indicated that neither total ApoE and ApoE4 levels nor the ApoE/ApoE4 ratio correlated with the diagnosis of AD. These findings reinforce the conclusions of previous studies in which plasma ApoE levels had no obvious clinical significance. *Molecular & Cellular Proteomics* 11: 10.1074/mcp.M112.018861, 1389–1403, 2012.

Apolipoprotein E (ApoE) is a 299-amino acid protein associated with lipoproteins in the plasma and the cerebrospinal fluid (1). The three major genetic variants of ApoE in the general population isoforms (E2, E3, and E4, encoded by the ϵ 2, ϵ 3 and ϵ 4 alleles, respectively) differ by a single amino acid: E2 (cys112, cys158), E3 (cys112, arg158), and E4 (arg112, arg158). Among the different susceptibility genes associated with the risk of late-onset Alzheimer's disease (AD)¹ and other neurological conditions, ApoE has been identified as a strong genetic determinant (1). Moreover, the risk of AD is strongly correlated with ApoE4 allele because the presence of one or two copies of the allele increases risk of late-onset AD by about three or 12 times, respectively. Furthermore, the presence of one or two copies of ApoE4 allele correlates with an earlier age of onset by about 10–20 years with regard to noncarriers in patients with late-onset disease (2–4).

The diagnostic value of an ApoE assay in AD is however subject to debate. Indeed, supranormal (5), subnormal (6),

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Received March 22, 2012, and in revised form, July 9, 2012

Published, MCP Papers in Press, August 23, 2012, DOI 10.1074/mcp.M112.018861

¹ The abbreviations used are: AD, Alzheimer's disease; ApoE, apolipoprotein E; ApoE4, isoform E4 of apolipoprotein; CV, coefficient of variation; ϵ 2, ϵ 3, ϵ 4, alleles 2, 3 and 4 of apolipoprotein E; LC, liquid chromatography; SID, stable isotope dilution; SRM, selected reaction monitoring; UHPLC, ultra high performance liquid chromatography.

and nondiscriminating (7–10) plasma concentrations of ApoE in AD cohorts have been reported in many studies based on immunoassay techniques. We wondered whether this ambiguity might be resolved by applying quantitative mass spectrometry (MS). Indeed, the combination of standardized, stable isotope dilution with targeted MS in selected reaction monitoring (SRM) mode is now widely accepted as a valuable alternative to immunoassays for accurate protein quantification (11–17) and may constitute the eagerly awaited bridge between the discovery and verification phases for candidate biomarker panels (18–22). A range of pilot studies have evaluated the sensitivity that can be achieved by SRM monitoring (23, 24); when tracking protein in a whole plasma hydrolysate, limits of quantification (LOQs) in the low $\mu\text{g/ml}$ range are frequently reported (12). However, LOQs in the low nanogram/ml range or the detection of proteins expressed with a low copy number per cell require either immuno-enrichment of the target protein or fractionation of proteotypic peptides by cation exchange (24, 25) or off-gel electrophoresis (26). A generic strategy based on stable isotope standards and capture by antipeptide antibodies (referred to as SISCAPA) is a promising alternative to more traditional enrichment strategies. Numerous reports have already demonstrated the ability of single or multiplexed immuno-SRM to achieve limits of quantification of 1 nanogram/ml or below when starting from less than 100 μl of biofluid (27). Precision, reproducibility and robustness are also key parameters in the development of SRM-based assay platforms dedicated to the clinical verification phase. Indeed, accuracy may even be a secondary consideration at this point, because the main objective is to discriminate between specific and nondiscriminating biomarker candidates within the clinical panel. Various pilot studies of the intra- and interclass performances of SRM monitoring have demonstrated acceptable coefficients of variation and levels of imprecision (below 20%) and, importantly, have pinpointed the main pitfalls that can distort assay results (28–33). Indeed, performance levels and reliability of an assay can be drastically compromised by intraclass variance within the proteotypic peptide fraction, which is caused by chemical modifications of certain amino acid side chains. In particular, peptides containing methionine residues are usually not recommended for SRM-based assays, because the sulfur atom is prone to oxidation (34–36) both *in vivo* (37, 38) and during sample handling or storage (giving rise to methionine sulfoxide). Unfortunately, peptides containing one or more methionine residues are sometimes the only ones available. By way of an example, 17% of the 625 proteins identified by only one peptide in Human Plasma PeptideAtlas database contain at least one methionine. Hence, quantification of this type of proteotypic peptide is challenging because the ratio between nonoxidized and mono-oxidized sulfoxide forms of heavy-labeled standards also changes during storage. To circumvent this limitation, complete oxidization of the methionine-containing peptide population with chemical oxidant has been

attempted but was not sufficiently robust or reproducible over several weeks in the context of large clinical study (39, 40).

Here, we report on the absolute quantification of total ApoE and ApoE4 isoform peptides by liquid chromatography (LC)-SRM targeted MS in a case-control study ($n = 669$). Because ApoE4-specific trypsin peptide possesses a single methionine residue (LGADMEDVR), oxidation was performed immediately after enzymatic digestion. The completeness of was carefully checked. We continuously assessed the overall performance of the assay by introducing quality control samples throughout the clinical cohort study, in order to ensure the validity of statistical comparisons between healthy controls and groups of AD patients.

EXPERIMENTAL SECTION

Standard Protocol Approvals, Registrations and Patient Consents—All subjects gave written informed consent for participation in this study. For those with substantial cognitive impairment, a caregiver, legal guardian, or other proxy gave consent. The study protocols were reviewed and approved by the appropriate Institutional review boards (CCPRB Brest, n°970-436-001 and CCPRB Lille, n°92/68).

Reagents and Chemicals—Acetonitrile, methanol and water (all LC-MS grade) were obtained from Fisher Scientific (Strasbourg, France). Dithiothreitol, iodoacetamide, ammonium bicarbonate, urea, formic acid, hydrogen peroxide, phenol and trypsin (type IX-S, from porcine pancreas) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Human plasma was obtained from the Institut Pasteur (Lille, France). Heavy peptide standards ($^{13}\text{C}_5$, ^{15}N on the valine residue) and light peptide derived from ApoE (LGPLVEQGR) and ApoE4 (LGADMEDVR) were synthesized by Millegen (Labège, France).

Enzymatic Digestion—Plasma samples (100 μl) were denatured in 6 M urea, 15 mM dithiothreitol at 60 °C for 40 min and then alkylated with 35 mM iodoacetamide at room temperature in the dark for 40 min. To reduce the urea concentration, the samples were diluted 5-fold with ammonium bicarbonate before overnight digestion at 37 °C with trypsin using a 1:30 (w/w) enzyme to substrate ratio. Digestion was stopped by addition of formic acid to a final concentration of 0.5%. Heavy peptides (LGPLV^{EQGR} and LGAMEDV^R) were then added.

Methionine Oxidation—Performic acid was freshly prepared by dissolving 25 mg of phenol in 4.5 ml of formic acid. After cooling the solution to 0 °C, 0.5 ml of hydrogen peroxide was added. The resulting solution was gently stirred at room temperature for 30 min. 1.25 ml of performic acid was then added to the digested plasma. Oxidation took place for 24 h at 4 °C.

Sample Desalting—Samples were desalted using OasisTM HLB 3cc (60 mg) reversed phase cartridges (Waters, Milford, MA). Before loading of the tryptic digest, the Oasis cartridges were conditioned with 1 ml of methanol and then 1 ml of water containing 0.5% formic acid. Cartridges were washed with 1 ml of methanol/water (5/95, v/v) containing 0.5% formic acid and eluted with 1 ml of methanol containing 0.5% formic acid. Samples were evaporated to dryness and resuspended in 100 μl of water containing 0.5% formic acid.

Liquid Chromatography-mass Spectrometry—The LC-MS/MS analysis was performed with an HP 1290 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP[®] 5500 LC-MS/MS hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo VTM ion source. Instrument control, data acquisition and processing were performed using the associated Analyst 1.5.1 software. The mass spectrometer was initially calibrated with a polypropylene glycol standard (AB

Sciex, Foster City, CA, USA). Q1 and Q3 quadrupole resolution was adjusted to 0.7 ± 0.1 Th. FWHM (referred to as unit resolution). The MS analysis was carried out in positive ionization mode, using an ion spray voltage of 5500V. The nebulizer (nitrogen) and the curtain gas flows (nitrogen) were both set to 50 psi. The Turbo V™ ion source was operated at 600 °C and the auxiliary gas flow (nitrogen) was set to 50 psi. The ultra-HPLC (UHPLC) separation was carried out on a Zorbax Eclipse plus C₁₈ column (50 mm x 2.1 mm, particle size 1.8 μm) from Agilent Technologies (Waldbronn, Germany). Elution was performed at a flow rate of 600 μl/min with water containing 0.1% (v/v) formic acid as eluent A and acetonitrile containing 0.1% (v/v) formic acid as eluent B. The elution sequence comprised a 1.3-min plateau with 8% B, a linear gradient from 8% B to 20% B in 1.2 min and, lastly, a 0.8 min plateau with 100% B. The column was re-equilibrated for 1.5 min and the overall injection duty cycle was less than 5 min. The column temperature was set to 25 °C. From the initial set of candidate SRM transitions, those tracking LGPLVEQGR (484.8/701.4; 484.8/588.3; 484.8/489.2) and doubly oxidized LGADM(ox-ox)EDVR (519.3/924.4; 519.3/867.4; 519.3/681.3) peptides were selected for the final assay. The collision energy, declustering potential, and collision cell exit potential were optimized for each chosen SRM transition by infusing synthetic peptides. The SRM transitions were monitored and acquired at unit resolution in Q1 and Q3, with dwell times of 15 ms and 21 ms used for each transition for the LGPLVEQGR and LGADMEDVR peptides, respectively (yielding over 25 data points for each peak on the chromatogram).

Determination of Natural Oxidation—Sixty-eight ε3ε4 plasma samples were digested and desalted as described above. For the non-oxidized LGADMEDVR peptide, the 503.3/892.4, 503.3/835.4, and 503.3/518.3 SRM transitions were monitored. For the mono-oxidized form, the SRM transitions were 511.3/908.4, 511.3/844.4, and 511.3/780.4. The HPLC separation was carried out on a Symmetry C₁₈ column (100 mm × 2.1 mm, particle size 3.5 μm) from Waters (Milford, MA, USA). Elution was performed at a flow rate of 300 μl/min with water containing 0.1% (v/v) formic acid as eluent A and acetonitrile containing 0.1% (v/v) formic acid as eluent B. Elution comprised a 3-min plateau with 5% B, a linear gradient from 5% B to 16% B in 8 min and, lastly, a 4-min plateau with 100% B. The column was re-equilibrated for 4 min. The overall injection duty cycle was 19 min and the column temperature was set to 25 °C.

Analysis of the Time Course of Methionine Oxidation—One hundred microliters of plasma were digested as described above. Heavy peptides (LGPLVEQGR and LGADMEDVR) were added to 1 ml of digested solution to a final concentration of 1.2 mg Apo E/ml plasma. For the pre-oxidation sample, 250 μl of water were added and solution was centrifuged at $15,000 \times g$ for 30 min at 15°C. The supernatant was recovered and 10 μl were injected into the HPLC system. Separation was performed under the same conditions as for determination of natural oxidation, except that the overall injection duty cycle was 30 min. For the oxidized samples, 250 μl of the mixture containing performic acid and phenol were added to another ml of digested solution containing the LGPLVEQGR and LGADMEDVR peptides at 1.2 mg Apo E/ml. The solution was centrifuged at $15,000 \times g$ for 30 min at 15°C. The supernatant was recovered and placed in an autosampler at 4°C. Ten microliters of solution were injected after 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 45 h.

Preparation of Standards and Quality Control Samples—Apolipoprotein E4 calibration curves ($n = 3$, for 3 days) were obtained by diluting ε4ε4 plasma into blank ε3ε3 plasma. The initial ApoE4 concentration was estimated to be 46.6 μg/ml in ε4ε4 plasma. Plasma standards and quality control (QC) samples were prepared by diluting ε4ε4 plasma into ε3ε3 plasma before enzymatic digestion. The ε4ε4 plasma volume ratios were 0, 0.14, 0.29, 0.43, 0.57, 0.71, and 1.0 for standards and 0.17, 0.51, and 0.86 for QC samples. Each standard

and QC sample was digested with trypsin separately, and then oxidized and desalted as described above.

Data Analysis—Mean plasma ApoE and ApoE4 concentrations were compared between each group by analysis of variance (ANOVA - F-Test) with the analysis tool pack of Microsoft Office Excel 2007 and p value < 0.05 was regarded as statistically significant. Data were then studied by principal component analysis (PCA) with Jump (version 9.0) software. To achieve the PCA, qualitative variables having n levels were converted into $n-1$ discrete quantitative variables coded 0 or 1. For example, male and female samples were encoded 0 and 1, respectively. The other three parameters used were the age of patients, their total ApoE and ApoE4 plasma concentration.

RESULTS

The primary objective of the study was to develop an SRM-mode, MS-based assay for total plasma ApoE and the ApoE4 isoform in a cohort of AD patients. The SRM mode is now considered to be a powerful and orthogonal alternative to enzyme immunoassays. However, very few examples of large-scale analyses ($n > 100$) over periods of weeks in true clinical cohorts have been published. Given the controversy over the value of ApoE levels in the diagnosis of AD, we evaluated not only conventional performance parameters (e.g. the interday ($n = 9$) CVs of six-point calibration curves, the linearity of the response and the LOQ) but also infrequently documented parameters, such as the stability of the labeled “heavy” peptide standards and the CV for the quality control samples over the weeks-long assay period. The sensitivity and specificity of SRM-based assays depend on appropriate selection of the proteotypic peptides. In our case, trypsin hydrolysis furnishes a single peptide (LGADMEDVR) for specifically assaying ApoE4, whereas LGPLVEQGR (previously identified as a good proteotypic candidate (12, 41, 42)) was monitored for total ApoE.

Levels of Mono-oxidized LGADMEDVR Peptide in the Plasma Hydrolysate—The signal from methionine-containing peptides is usually split into a nonoxidized/oxidized doublet as the combined result of natural and artificial oxidation (38). We thus checked the relative level of the oxidized form of the ApoE4 LGADMEDVR peptide sequence in ε3ε4 plasma. Product ion mass spectra of doubly protonated molecular ions of mono-oxidized and nonoxidized LGADMEDVR endogenous peptide (Fig. 1A and 1C) were used for identification and the subsequent choice of the best SRM transitions. The main observed peaks arose from y fragment ions and confirmed both the peptide sequences and the methionine residue oxidation state. In the case of mono-oxidized peptides (see the MS/MS spectrum in Fig. 1A), these y ions eliminate the side chain of the methionine residue and yield fragment ions with a -64 Da shift. For each unambiguously attributed peptide sequence, three SRM transitions for the most intense MS/MS fragment ions were selected. Fig. 1B shows extracted ion chromatograms of SRM transitions 511.3/908.4 (blue) and 514.3/914.4 (red) for mono-oxidized, endogenous LGADM(ox)EDVR and for heavy LGADM(ox)EDVR and transitions 503.3/892.4 (green) and 506.3/898.4 (gray) for nonoxi-

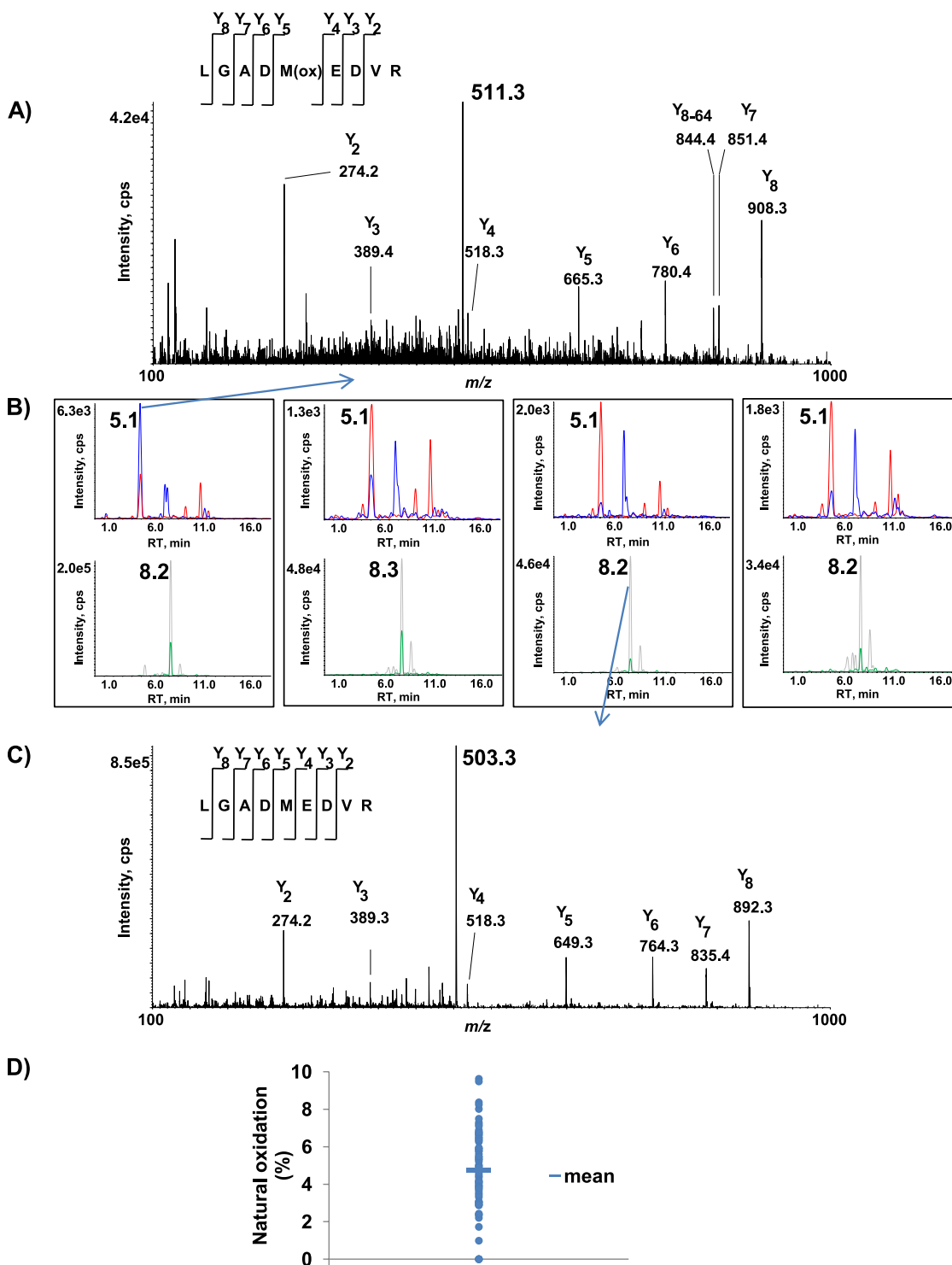


FIG. 1. Detection of endogenous oxidized and nonoxidized forms of the ApoE4-specific peptide LGADMEDVR in plasma from an AD cohort. *B*, shows extracted ion chromatograms of SRM transitions 511.3/908.4 (blue) and 514.3/914.4 (red) (monitored for mono-oxidized endogenous LGADM(ox)EDVR and heavy LGADM(ox)EDV'R) and transitions 503.3/892.4 (green) and 506.3/898.4 (gray) (monitored for nonoxidized endogenous LGADMEDVR and heavy LGADMEDV'R) in eight plasma samples. The MS/MS spectra correspond to the doubly protonated mono-oxidized endogenous LGADM(ox)EDVR ion (*A*) and the doubly protonated nonoxidized endogenous LGADMEDVR ion (*C*). Sixty-eight plasma samples from ε4 allele-bearing subjects (100 μl each) were selected randomly from the AD cohort and digested by trypsin before monitoring SRM transitions 511.3/908.4 and 503.3/892.4 (tracking oxidized and nonoxidized ApoE4-specific peptide LGADMEDVR, respectively) (*D*).

dized endogenous LGADMEDVR and heavy LGADMEDVR in eight plasma samples. The results clearly show that the mono-oxidized species (detected at RT = 5.1 min) and the nonoxidized form (detected at RT = 8.2 min) are present in ε 3 ε 4 plasma. Even the mono-oxidized form of the LGADMEDVR peptide standard was detected. Fig. 1D shows the oxidation ratio across a panel of 68 ε 3 ε 4 plasma samples (assuming that the ionization yield and the matrix effect are similar for the two species). The proportion of the oxidized form ranged from 0% to 10% and thus highlighted the inter-sample heterogeneity in endogenous oxidation.

The Time Course of Methionine Oxidation—The presence of both non- and oxidized peptides in almost all plasma samples not only decreases sensitivity but also prevents accurate quantification of the ApoE4 isoform. In order to eliminate this source of bias, we chose to convert the ApoE4-specific LGADMEDVR peptide into the sole doubly oxidized methionine-containing species before MS quantification. The oxidation yield was initially assessed by monitoring the synthetic heavy LGADMEDVR peptide standard spiked in water (Figs. 2A–2C). As expected, the full mass spectrum of the AQUA peptide LGADMEDVR (Fig. 2A) shows a nonoxidized form (the doubly protonated $[M+2H]^{2+}$ ion at m/z 506.2) and a mono-oxidized form (the doubly protonated ion $[M+O+2H]^{2+}$ at m/z 514.3). We then evaluated hydrogen peroxide oxidation for 20h at 4 °C (Fig. 2B). Even though the doubly oxidized species was well detected as the major product (with a doubly protonated $[M+2O+2H]^{2+}$ ion at m/z 522.2), the mono-oxidized form (m/z 514.3) was nevertheless still present. We then looked at whether a performic acid/phenol mixture could increase the oxidation yield. Phenol has been shown not to oxidize tyrosine residues, which may be of interest for future work on proteotypic peptides containing both tyrosine and methionine residues (43). As shown in Fig. 2C, the oxidation of the methionine residue in the LGADMEDVR peptide was stoichiometric and yielded the $[M+2O+2H]^{2+}$ ion alone (detected at m/z 522.2). Given that the LGPLVEQGR peptide is also monitored (to assay total ApoE), we also assessed the stability of this peptide to oxidation. Figs. 2D–2F shows the MS spectra of the synthetic LGPLVEQGR peptide standard in water under the different oxidation conditions. The fact that only the doubly protonated ion $[M+2H]^{2+}$ was ever detected (at m/z 487.8) demonstrated the lack of sensitivity of the peptide to oxidation.

The time course of methionine oxidation was further studied by spiking both LGADMEDVR and LGPLVEQGR AQUA standards at a concentration of 1.2 mg/ml in digested plasma, to evaluate a potential matrix effect that could have affected the oxidation yield. Fig. 2G shows the SRM signal (blue) that tracks the kinetics of oxidation of the LGADMEDVR into the doubly oxidized species by the performic acid/phenol mixture (see supplemental Fig. S1 in the supplemental materials for the MS-MS spectra that defines the three most intense transitions) and the stability of LGPLVEQGR. The blue line high-

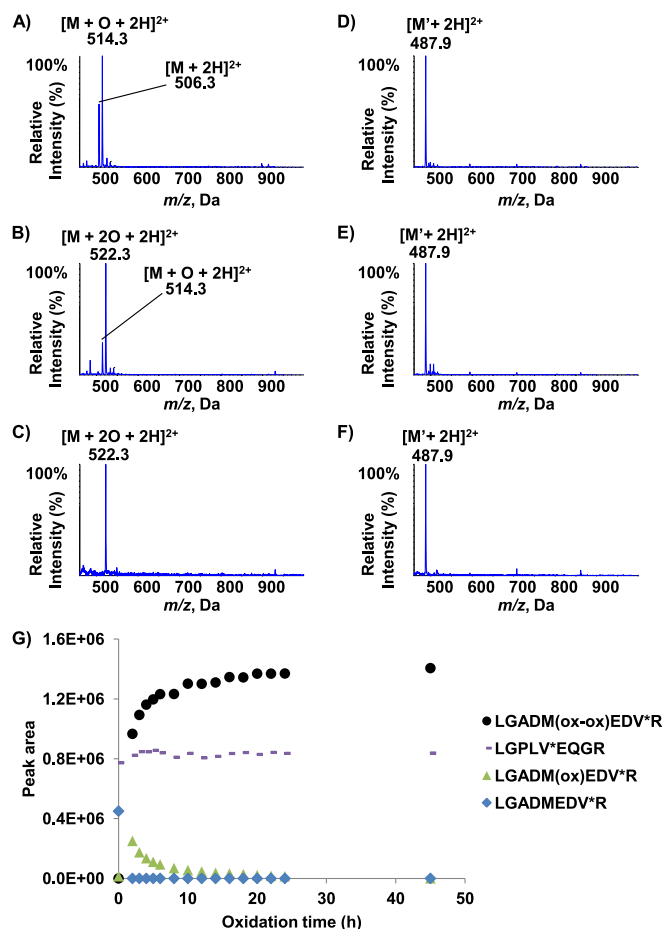


Fig. 2. Full mass spectra of AQUA peptides LGADMEDVR and LGPLVEQGR after oxidation in water and Oxidation kinetics in plasma. Mass spectra of synthetic LGADMEDVR peptide in water containing 0.5% formic acid (A), after oxidation with hydrogen peroxide (B) and after oxidation with performic acid and phenol (C). Mass spectra of synthetic LGPLVEQGR peptide in water containing 0.5% formic acid (D), hydrogen peroxide (E) or performic acid and phenol (F). G, shows the change over time in the LGADMEDVR (blue), LGADM(ox)EDVR (green), LGADM(ox-ox)EDVR (black) and LGPLVEQGR (pink) peak areas during oxidation with a performic acid/phenol mixture in digested plasma.

lights the rapid oxidation kinetics, because the nonoxidized species is already no longer detectable at $t = 2$ h. The evolution of the disappearance of the mono-oxidized LGADM(ox)EDVR (green) corresponds to the appearance of the doubly oxidized LGADM(ox-ox)EDVR (black) species. The signal curve of the latter indicates that the irreversible, double-oxidation reaction (38) was complete by 15h—even though the reaction was extended to 24 h for convenient time management in the clinical sample workflow. The pink curve emphasizes the high stability to oxidation of the LGPLVEQGR proteotypic peptide used to track all three ApoE isoforms. The stoichiometric conversion of endogenous (light) nonoxidized and mono-oxidized LGADMEDVR peptide sequence into a single, doubly oxidized species was also assessed in a sample of pooled plasma containing APOE4 isoform.

Supplemental Fig. S2 shows that, as expected, the nonoxidized and mono-oxidized LGADM(ox)EDVR peptides are detected before oxidation (at RT = 8.1 min and RT = 4.2 min, respectively (supplemental Fig. S2A and S2B), whereas the doubly oxidized LGADM(ox-ox)EDVR peptide is not (supplemental Fig. S2C). After 24 h of oxidation, only the doubly oxidized LGADM(ox-ox)EDVR peptide remains detectable (at RT = 6.5 min) in the plasma sample, demonstrating the completeness of the reaction for the endogenous LGADMEDVR peptide.

Validation of the Method and Cohort Analysis—Documented measurement accuracy is essential for the approval of biomarkers by the regulatory agencies but may be less important during the clinical evaluation phase, the ultimate goal of which is to distinguish between specific and nonspecific candidates. However, the quantification has to be repeat-

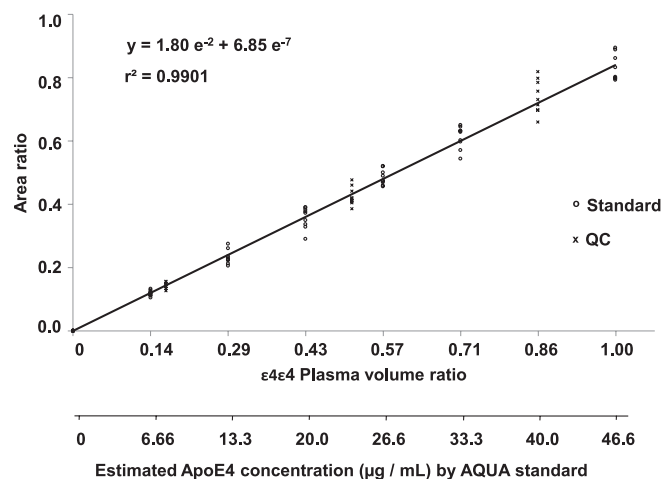


FIG. 3. Calibration curve for doubly oxidized endogenous LGADM(ox-ox)EDVR. The peak area ratio between the doubly oxidized endogenous LGADM(ox-ox)EDVR and the doubly oxidized internal standard LGADM(ox-ox)EDVR is plotted against the dilution of ε4ε4 plasma in ε3ε3 plasma over a three-day experiment (three replicates per day). The heavy LGADMEDVR peptide standard was spiked at a constant concentration of 55.6 μg/ml equivalent to ApoE4 protein.

able. The debate on whether a heavy-labeled protein standard (which supposedly compensates for incomplete trypsin digestion) or AQUA peptide standard should be used was not applicable to our present study. Other groups have documented a high digestion yield and a linear response for ApoE in human plasma (12, 41, 42); this was confirmed by our present findings. Furthermore, the concentrations measured here were within the range of values reported in the literature for ELISAs.

Given the size of our AD cohort, the final assay was developed in an ultra-high performance liquid chromatography format. The overall cycle time for injection, separation, and conditioning was ~5 min per analysis. This enabled us to process 80 samples per night *i.e.* two separately processed series for 40 patients. Because this type of chromatographic format is more subject to a matrix effect (because of a lower peak capacity), precision and robustness parameters were carefully evaluated to guarantee confident final statistical analysis. The overall analytical variability of the full sample workflow (including methionine oxidation) for three experiments over a one-week period and three experimental replicates per day was calculated as the coefficient of variation (CV). Each experiment comprised three replicates of six different dilutions of ε4ε4 plasma into ε3ε3 plasma (to cover the expected range of ApoE4 concentrations) and a spiked AQUA standard at a fixed concentration (Fig. 3). We also constituted quality controls at three different dilutions. Linear calibration curves were obtained by plotting peak area ratio between the LGADM(ox-ox)EDVR and the LGADM(ox-ox)EDVR peptides *versus* the volume ratio of ε4ε4 plasma in ε3ε3 plasma. The peak area of endogenous LGADM(ox-ox)EDVR peptide (reconstituted on the most intense 519.3/924.4 SRM transition) was corrected by the peak area of the stable-isotope labeled peptide (tracked by transition 522.3/930.4). Linearity was observed between dilution factors 1 to 7, which corresponded to calculated concentrations (according to AQUA peptide standardization) of 46.6 to 6.7 μg/ml ($r^2 > 0.99$). The ratio between nominal and calculated concentrations was below 15% for

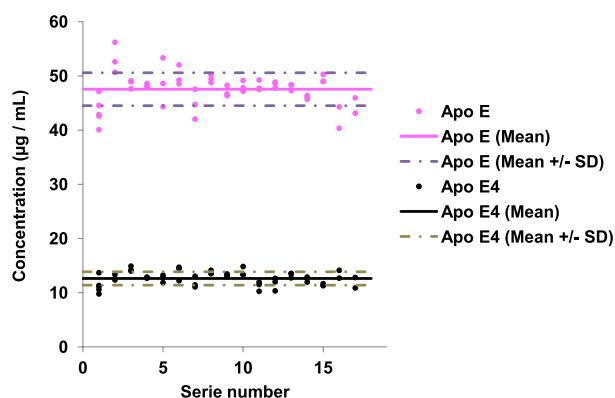
TABLE I

Analytical performance levels (precision and accuracy) calculated for replicated calibration curves of ApoE4 peptide in ε4ε4 plasma diluted with ε3ε3 plasma

Sample type	n	ε4ε4 plasma volume ratio	Estimated concentration (μg/ml)	CV (%) ^a	Accuracy ^b
Standard	9	0.00	0.0	4.8	N/A
Standard	9	0.14	6.66	7.2	99.2
Standard	9	0.29	13.3	9.5	97.3
Standard	9	0.43	20.0	9.5	99.6
Standard	9	0.57	26.6	5.0	101.2
Standard	9	0.71	33.3	5.8	102.0
Standard	9	1.00	46.6	5.0	98.9
QC	9	0.17	7.99	6.6	99.7
QC	9	0.51	24.0	6.8	98.6
QC	9	0.86	40.0	7.2	102.9

^a Expressed as the relative standard deviation (S.D.), *i.e.*, S.D./mean × 100.

^b Expressed as a percent difference: ((concentration found – concentration added)/concentration added) × 100.



	Apo E	Apo E4
mean +/- SD (µg / mL)	47.6 +/- 3.0	12.6 +/- 1.2
Precision (%)	6.4	9.7

FIG. 4. **Quantification of total ApoE and ApoE4 in quality control samples.** Mean and mean \pm standard deviation values for the concentration of total ApoE and doubly oxidized ApoE4-specific peptide in the 17 series of triplicated quality control samples ($n = 51$) run throughout the clinical cohort study.

99% of standard and QC samples, as reported in Table I (see also supplemental Table S1). Within three process replicates, the relative standard deviation and accuracy were between 4 and 10% and 97 and 103%, respectively. The response linearity and variability of LGPLV^EEQGR standard diluted in digested plasma were assessed in a similar way (see supplemental Fig. S3).

As recommended in regulatory agency guidelines, the interday variation of the full analytical process was systematically assessed by introducing triplicate QCs into each of the 17 series of 40 patients randomly selected from the 669-strong cohort. The 8 ml pool of plasma used for QC samples was obtained by pooling 200 μ l aliquots of plasma from 40 patients into 8 tubes. A statistical analysis of QC sample assays (Fig. 4) run throughout the cohort study showed a mean imprecision below 10% for both total ApoE and ApoE4 isoform quantification and no individual imprecisions that exceeded the mean calculated concentrations (mean total ApoE concentration: 47.6 ± 3.0 μ g/ml; mean ApoE4 concentration: 12.6 ± 1.2 μ g/ml) by more than 20%. These controls illustrated the good repeatability of the full analytical process and the reasonable degree of confidence for analyzing the control patient cohort ($n = 221$) and the clinically diagnosed AD patients ($n = 451$). Fig. 5 shows typical extracted ion chromatograms obtained during the cohort analysis for SRM transitions 519.3/924.4 (blue), 519.3/867.4 (red) and 519.3/681.3 (green) (monitored for the doubly oxidized endogenous LGADM(ox-ox)EDVR) and transitions 522.3/930.4 (gray), 522.3/873.4 (orange) and 522.3/687.3 (black) (monitored for the doubly oxidized heavy LGADM(ox-ox)EDV^R) in ϵ 4 ϵ 4 plasma (A), ϵ 3 ϵ 4 plasma (B), and ϵ 3 ϵ 3 plasma (C) samples.

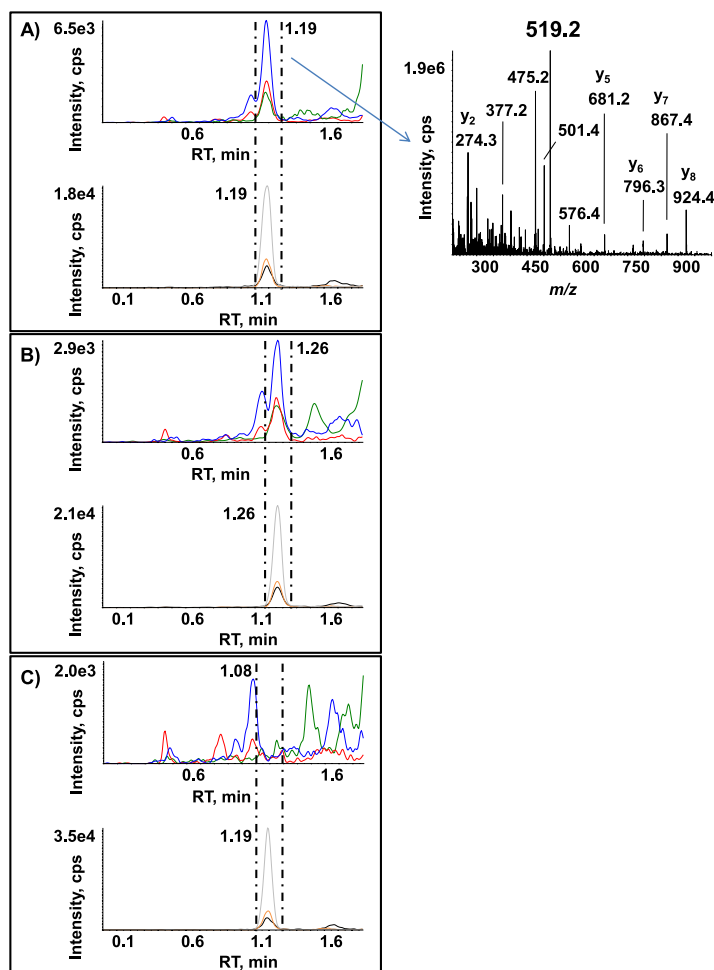
Both doubly oxidized endogenous LGADM(ox-ox)EDVR and heavy LGADM(ox-ox)EDV^R peptides were detected at RT = 1.19 min and RT = 1.26 min from ϵ 4 ϵ 4 and ϵ 3 ϵ 4 plasma, respectively. As expected, no doubly oxidized endogenous LGADM(ox-ox)EDVR was detected at RT = 1.19 min (the heavy standard) for the ϵ 3 ϵ 3 plasma, because of the absence of ApoE4 in this genotype; this emphasizes the specificity of detection.

The total ApoE and ApoE4 levels were determined for each sample and results are reported in supplemental Table S2. Fig. 6 shows total ApoE and ApoE4 levels (except for the genotypes ϵ 2 ϵ 3 and ϵ 3 ϵ 3 lacking ApoE4) determined from the control (blue) and AD (red) samples for the ϵ 2 ϵ 3 (A), ϵ 3 ϵ 3 (B), ϵ 2 ϵ 4 (C), ϵ 3 ϵ 4 (D), and ϵ 4 ϵ 4 (E) genotypes. The total ApoE concentration in plasma sample was around 50 μ g/ml, which is consistent with the literature values (8–10). The total ApoE and ApoE4 levels varied according to the individual's genotype (as also reported in the literature (10)) in the following order: ϵ 2 ϵ 3 > ϵ 2 ϵ 4 > ϵ 3 ϵ 3 > ϵ 3 ϵ 4 > ϵ 4 ϵ 4 (see supplemental Table S3 for the mean values from AD samples for each genotype). An analysis of variance (ANOVA) yielded p values <0.05, indicating that the inter-genotype difference are statistically significant. For ϵ 3 ϵ 4 heterozygous patients, the ApoE4 protein only accounted for between 30 and 40% of the total ApoE (rather than 50%) (44). However, the control and AD groups did not differ significantly in this respect. Table II summarizes the total ApoE and ApoE4 levels obtained from the control and AD samples and the ANOVA results for each genotype. As indicated by the p values >0.05, the control and AD samples did not differ significantly in terms of total ApoE or ApoE4 levels. The data on ApoE and ApoE4 levels were further evaluated in a multivariate PCA. The parameters available for the samples (*i.e.* gender, age, genotype and three single nucleotide polymorphisms (SNP) (cf supplemental Table S2)) were chosen as descriptive variables and ApoE and ApoE4 levels were used as the quantitative variables. Fig. 7 shows the PCA plot for the ApoE level (Fig. 7A) and ApoE4 level (Fig. 7B) data sets, with the control (blue triangles) and AD (red circles) samples for ϵ 3 ϵ 3 (Fig. 7A) and ϵ 3 ϵ 4 (Fig. 7B) genotypes. Principal component 1 mostly depends on the SNP(427) factor, whereas principal component 2 depends on the SNP(419) factor. Principal component 1 and 2 accounted for about 45% of the total data variability. In conclusion, our analysis of total ApoE and ApoE4 isoform concentrations in plasma emphasized these parameters' inability to discriminate between our control and AD populations. However, ANOVA results confirm that the A/T transversion at nucleotide 491 significantly decreases plasma ApoE concentration independently of genotype or disease: 58.0 ± 16.2 μ g/ml ($n = 198$) and 52.8 ± 12.3 μ g/ml ($n = 73$) ($p = 0.012$).

DISCUSSION

Artifacts of methionine mono-oxidation during sample storage and preparation is especially harmful, because it pre-

FIG. 5. Reconstructed ion chromatograms for transitions monitoring doubly oxidized endogenous LGADM(ox-ox)EDVR and AQUA LGADM(ox-ox)EDV'R peptide sequences. The figure shows an overlay of SRM transitions 519.3/924.4 (blue), 519.3/867.4 (red), and 519.3/681.3 (green) (monitored for the doubly oxidized endogenous LGADM(ox-ox)EDVR) and SRM transitions 522.3/930.4 (gray), 522.3/873.4 (orange) and 522.3/687.3 (black) (monitored for the doubly oxidized heavy LGADM(ox-ox)EDV'R) in samples of $\epsilon 4\epsilon 4$ plasma (A), $\epsilon 3\epsilon 4$ plasma (B) and $\epsilon 3\epsilon 3$ plasma (C). The MS/MS spectrum corresponds to the doubly protonated, doubly oxidized endogenous LGADM(ox-ox)EDVR ion.



vents accurate, direct measurements of the natural oxidation level. Before the present study, we had carried out work that systematically evaluated the influence of storage conditions on the extent of oxidation of ApoE4 AQUA peptide; this parameter was especially influenced by the number of freeze-thaw cycles (data not shown). Hence, recently published articles on SRM-based protein quantification have generally not recommended methionine-containing proteotypic peptides as good candidates for the clinical validation of putative protein biomarkers (34–36). The relevance of this advice is borne out by the fact that the proportion of mono-oxidized ApoE4 proteotypic peptide ranged from 0% to 10% in our present clinical AD cohort. This aspect was not considered in a previous report on simultaneous quantification of ApoE isoforms by tandem MS (45), even though the presence of the oxidized form must have considerably biased the final result.

Our present study showed that stoichiometric oxidation of methionine by phenol/performic acid mixture before MS quantification is a valuable way of circumventing this difficulty. In their pioneering AQUA article, Gygi's group applied a similar strategy for quantification of *in vitro* phosphorylated isoforms of separase by a bank of kinases (39). Very recently,

Huillet *et al.* also used methionine oxidation before the quantification of troponin I (40). However, the method's performance levels and robustness were not evaluated in enough detail for its reliable transfer to large clinical studies.

In the present work, we have optimized and validated a sample workflow (with an overall CV below 10%) that includes protein digestion, methionine oxidation, desalting, and UHPLC-SRM-MS monitoring. This performance was calculated for three interday experiments, each of which comprised three calibration curves run in triplicate and drawn from six dilution points of $\epsilon 4\epsilon 4$ plasma into $\epsilon 3\epsilon 3$ plasma (covering the clinical concentration range for ApoE4). Similar statistics (again with a CV < 10%) were obtained for all triplicates of the QC samples introduced in each of the 17 series of 40 patient samples per run throughout the entire analysis of the AD cohort and the control subjects ($n = 669$). The performance levels of the assay were reinforced by AQUA standardization and the use of internal QC samples (especially for assessing the day-to-day variability in digestion). The CVs were below 10% and were calculated for the 17 QC series analyzed over a period of two months (during which time other unrelated assays were also being run); these data highlight the robust-

ness and reproducibility of the full sample processing and analytical platform. These performance levels can be achieved as long as key experimental steps are included in the validation phase. The long-term stability of stock or working solutions of AQUA peptides or heavy-labeled protein standards should also be checked and documented, because it represents a potential major cause of variations. One could consider labeled (heavy) protein to be the gold standard, because it will compensate for any losses during sample

fractionation or for a poor digestion yield. Although the prospect of greater accuracy (relative to the AQUA method) with protein standards is attractive, the technique is likely to be less reliable; Hoofnagle's group recently stated that purified lipoproteins spiked into serum were not digested with the same efficiency as their native endogen counterparts (46).

We thus believe that QC samples (in pooled plasma aliquots, in the present case) should systematically be introduced into clinical cohort investigations, as recommended by the authorities for drug evaluation. As very recently mentioned by Kuhn *et al.*, instrumental calibration may also be involved in dramatically degraded assay performance by shifting the isolation window for both precursor and product ions (31). Hence, before each new analytical run, we always assessed instrumental performance levels (sensitivity and calibration) by monitoring the ApoE common synthetic peptide.

To the best of our knowledge, our clinical cohort was one of the largest to have been screened with MS-based protein assays. It is also important to highlight the relative high plasma concentrations of ApoE studied here, relative to the ng/ml or even sub-ng/ml values expected for the leakage of organ-specific tumor biomarkers. There is no evidence to suggest that methionine oxidization would not be quantitative for minor proteins or that it could not be combined with peptide enrichment. It is noteworthy that in proteomics, methionine oxidization has already reliably furnished relative quantification data for the discovery of candidate biomarkers of oxidative stress targets (47–49). Notwithstanding the relatively high concentration of ApoE in plasma, we believe that our present management of such a large cohort of samples unequivocally contributes to the convincing demonstrations that multiplexed SRM protein assay is of great value for clinical evaluation of candidate biomarkers.

However, whether SRM could not only serve for up-stream evaluation studies but also be a platform for clinical assays is debatable. There are two underlying questions: 1) is the testing performance and throughput achieved by SRM sufficient regarding all requirements of routine analysis and 2) is SRM a cost effective technique. The question should even be formulated differently as it is first necessary to distinguish between

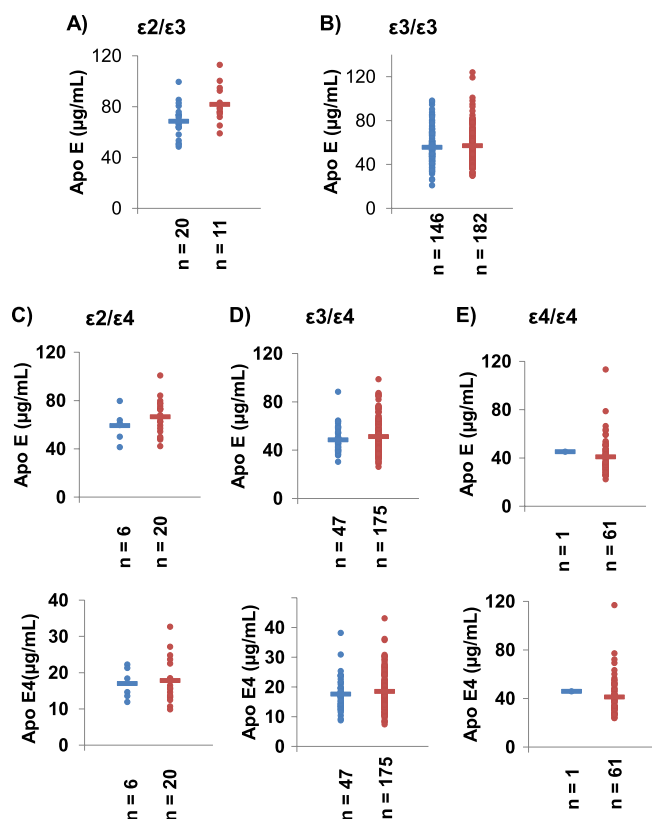


FIG. 6. Total ApoE and ApoE4 levels measured in the clinical AD cohort. Total ApoE concentrations in $\epsilon 4$ -lacking carriers of $\epsilon 2\epsilon 3$ (A) and $\epsilon 3\epsilon 3$ (B) alleles in the healthy control group (blue) and the AD cohort (red). Total ApoE (top) and ApoE4 isoform (bottom) concentrations for carriers of $\epsilon 2\epsilon 4$, $\epsilon 3\epsilon 4$ (D) and $\epsilon 4\epsilon 4$ (E) alleles in the healthy control group (blue) and the AD cohort (red).

TABLE II

ApoE and ApoE4 levels (mean \pm standard deviation) as a function of the genotype in healthy controls and diagnosed AD patients

Group	Genotype	n	Apo E (mean \pm S.D.)	p value	Apo E4 (mean \pm S.D.)	p value
Control	E2E3	20	68.5 \pm 13.0	0.065		
AD		11	77.3 \pm 10.5			
Control	E2E4	6	59.3 \pm 13.0	0.292	17.0 \pm 4.3	0.838
AD		21	66.2 \pm 14.2		17.5 \pm 5.8	
Control	E3E3	146	55.6 \pm 14.5	0.394		
AD		182	57.1 \pm 16.5			
Control	E3E4	48	48.3 \pm 10.5	0.157	17.4 \pm 5.6	0.269
AD		176	51.3 \pm 14.0		18.5 \pm 6.0	
Control	E4E4	1	45.2	0.780	45.9	0.771
AD		61	41.0 \pm 15.1		41.2 \pm 15.7	

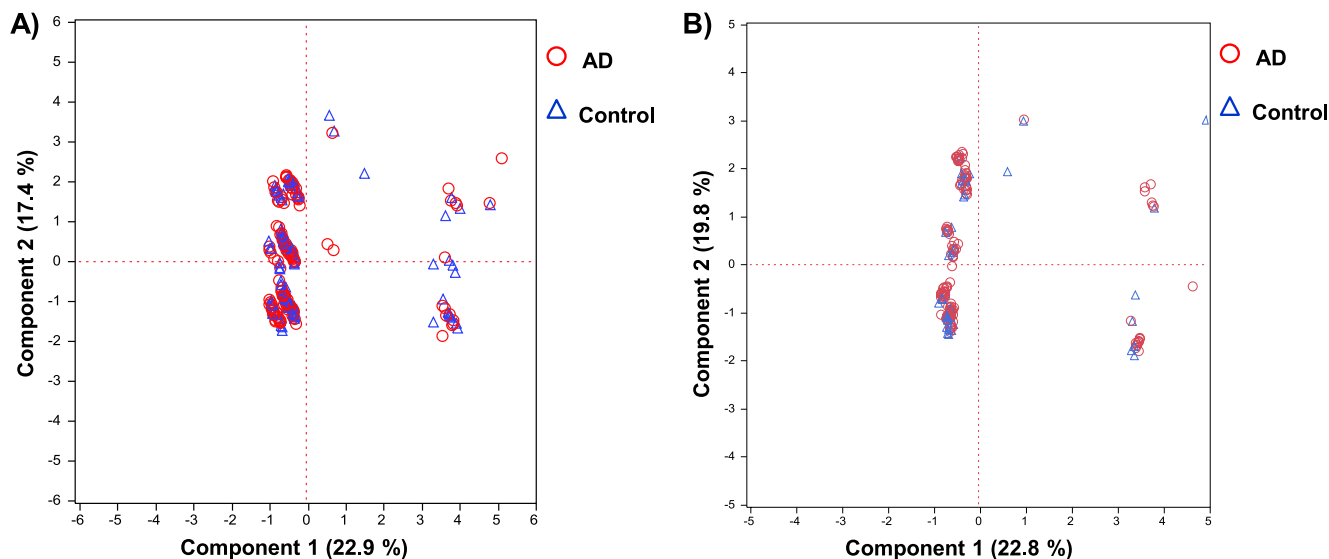


FIG. 7. A principal component analysis plot of the ApoE (A) and ApoE4 (B) concentration data sets, showing healthy control samples (blue triangles) and AD cohort samples (red circles) for $\epsilon 3\epsilon 3$ (A) and $\epsilon 3\epsilon 4$ (B) genotypes.

two types of biomarkers: those which may be directly quantified in plasma or serum without depletion and/or fractionation and those present at weak concentrations (below 100 ng/ml) that required depletion and/or peptide fractionation or enrichment. For the first category of highly abundant proteins, the recent studies by Hoofnagle's group back up this point of view. This group has demonstrated a reliable clinical quantification of apoA-I and apoB using a single-point calibration method, interassay CVs < 12% and excellent correlation ($r^2 > 0.82$) with the corresponding nephelometric immunoassays. More recently, they have rigorously compared two standardization methods based on isotope-dilution in the context of a six-plex apolipoprotein assay and compared the SRM assays with established immuno-assays (50). The results showed a relatively similar performance for the two isotope-dilution standardization methods, imprecision values < 11% and strong correlations with respect to the IA results. The recent work by Lassman *et al.* also focused on apolipoprotein quantification by SRM using AQUA standardization and reached the same interassay performance over 2 weeks (51), with CVs better than 12% and r^2 of 0.87 and 0.94 relative to immuno-turbidimetric assays for ApoB and ApoA, respectively. Note that this assay was configured in the same way as (like) ours with a UHPLC format enabling more than 100 analyses per day.

Besides precision, the robustness over weeks (or months) is another key issue, whatever the testing method, to go from a discovery or verification tool to a routine analysis method. Interestingly, the two above studies dealing with apolipoprotein quantification were performed either with micro flow or normal flow chromatography. The group of Borchers has very recently made judicious comparisons of the analytical performance of normal flow (2 mm i.d. columns) with nanoflow chromatography (52). The authors concluded with evidence of

increased robustness of the normal flow system, including improved retention-time stability with a mean CV < 0.5% for normal flow against a mean CV of 6% for nanoflow. Interestingly, and contrary to the common view, they have also shown that normal flow was on average more sensitive than nanoflow.

For the category of weakly concentrated proteins, such as biomarkers leaking from tumors in the nanogram/ml plasma range, clinically relevant low limits of quantification/detection are only reached after complex peptide fractionation by SCX (24) or immuno-enriching the target compound at the protein (53) or proteotypic peptide level (54, 55). Despite possible new sources of imprecision introduced by these additional steps, still acceptable intra- and inter-laboratory CVs usually below 12% were obtained at mid-point of the linear range even after applying peptide fractionation or immuno-enrichment (28, 29, 31, 32).

Together, these studies and the present work demonstrate, from our point of view, that well standardized sample workflows (trypsin digestion of whole plasma, serum or urine without any fractionation) associated with conventional or micro-flow systems achieve the required performance for today let envisage the possibility of developing clinical multiplex SRM assays.

Concerning the second question, whatever the most sensitive and efficient testing method, its application in a clinical routine utilization will always be greatly impacted by the cost of an analysis. For the first category of highly abundant proteins compatible with high throughput analysis by UHPLC, hence optimized instrument-time, the cost of the triple quadrupole instrument can no longer be considered a limiting factor because mid-sensitivity instruments are about 150,000 \$, only slightly more expensive than conventional automated testing devices. Furthermore, the global cost of all reagents

and heavy standards required per SRM-based assay could be estimated at about 1 \$ per testing point) and is finally low compared with consumables required for IA. At this time, it appears that for weakly abundant proteins (below 1 $\mu\text{g/ml}$), only the SISCAPA strategy would reach the required throughput and multiplexing capabilities.

The reliability of our combined quantification of total ApoE and the ApoE4 isoform is strengthened by its agreement with previous published data on nephelometric, turbidimetric, and immunoenzymatic assays. First, our SRM-based assay indicated total plasma ApoE concentrations ranging from 41 to 77 $\mu\text{g/ml}$ (depending on the genotype), in line with the range of concentrations commonly reported in the literature (*i.e.* 25 to 75 $\mu\text{g/ml}$ (5, 8–10, 44, 56–60)). This result emphasizes that when AQUA standardization is combined with simplified sample processing (and in the absence of protein fractionation, which is a major source of loss), the accurate measurement of medium-abundance plasma proteins is eminently possible. Another often-noted source of inaccuracy relates to inter-protein differences in sensitivity to trypsin hydrolysis (30); this problem may be partly circumvented by the use of isotopically enriched protein standards (61). Borcher's group classified ApoE as belonging to the rapidly digested family of plasma proteins, because 1) there were no significant differences in the digestion efficiency according to the denaturation protocol and 2) digestion was complete after 4h (42). This group estimated that the digestion yield was 100%, which supports the agreement between our SRM results and the ELISA results in the literature. Only two groups have measured significantly higher levels of circulating total ApoE in plasma (ranging from 120 to 180 $\mu\text{g/ml}$) (6, 44). Both these studies used the same ELISA kit, suggesting the presence of discrepancies in terms of detection specificity and/or inadequate standard quality.

Recent studies into the relationship between the ApoE genotype and plasma ApoE levels have evidenced higher ApoE levels in $\epsilon 2$ allele carriers and lower levels in $\epsilon 4$ allele carriers. Once again, the results of our SRM-based assay agreed well with previous findings. The reliability of the assay was also evidenced by our observation of higher concentrations of both total ApoE and the ApoE4 isoform in female subjects than in male subjects (supplemental Fig. S4).

The second important conclusion is the perfect agreement between our assays and previous findings concerning the relationship between the ApoE genotype and mean plasma ApoE level (10). Corresponding studies have indeed concluded with a higher mean level in $\epsilon 2$ allele carriers, next in $\epsilon 3$ and finally, the mean lowest level in $\epsilon 4$ allele carriers. This was confirmed by our observations of the influence of the genotype on total ApoE concentration in the following order $\epsilon 2\epsilon 3 > \epsilon 2\epsilon 4 > \epsilon 3\epsilon 3 > \epsilon 3\epsilon 4 > \epsilon 4\epsilon 4$.

The third piece of evidence proving the reliability of our SRM-based assay is the observed higher concentrations of total ApoE and the ApoE4 isoform in female subjects com-

pared with male subjects (supplemental Fig. S4). This is in line with previous reports that showed a higher mean ApoE concentration in plasma of females older than 50, likely related to hormonal changes after menopause (62, 63).

AD risk has also been reported as being associated with ApoE promoter polymorphisms. The three main characterized polymorphisms are located before the transcription start site: an A/T transversion at nucleotide 491, a T/C transition at nucleotide 427, and a T/G transversion at nucleotide 219 (64, 65). These polymorphisms are closely related to the ApoE promoter activity. Indeed, a single A to T base substitution at nucleotide 491 caused a significant decrease in APOE promoter activity whereas a T to G substitution at nucleotide 219 provoked the opposite effect in the human cell line HepG2 (hepatoma). On the other hand, a T to C substitution at nucleotide 427 had no effect on promoter activity (64). These modulations in promoter activity have a direct impact on the ApoE concentration in plasma or in cerebrospinal fluid because higher ApoE levels have been reported in these fluids when promoter activity is enhanced (*i.e.* when there is no substitution at nucleotide 491 or when there is a T to G substitution at nucleotide 219) (66–69). Some studies have indicated that the presence of adenine or thymine in position 491 and 219 respectively increased risk for AD (68–75). Although we did not find any difference at the ApoE concentration level between control and AD cohorts whatever the promoter polymorphism and genotype, we confirmed however the influence of A/T transversion at nucleotide 491 on total ApoE concentration ($58.0 \pm 16.2 \mu\text{g/ml}$ ($n = 198$) versus $52.8 \pm 12.3 \mu\text{g/ml}$ ($n = 73$)). This observation is a new and definitive illustration of the reliability of our SRM-based assay.

The exact role of apolipoprotein E protein (ApoE) in the development of AD is still not understood. Different work suggests that, besides exerting a qualitative effect on the occurrence of AD through variations in APOE $\epsilon 2/\epsilon 3/\epsilon 4$ coding polymorphisms, the quantitative expression of APOE may also be a key disease determinant (76, 77). Indeed, there is strong proof that ApoE isoforms modulate accumulation of toxic amyloid β ($A\beta$) oligomers in the brain, a decisive event in the pathogenesis of AD. For instance, in animal models expressing different human ApoE isoforms, the rate and kinetics of amyloid β oligomers are depend on the allele following the order $\epsilon 4 > \epsilon 3 > \epsilon 2$ (78–81). The same influencing order is found for brain amyloid β accumulation across cognitively normal individuals as demonstrated by imaging studies (82–85). Thus, a large body of evidence converges on a pivotal role of ApoE in the neurodegeneration process associated with a possible disruption of ApoE levels in the cerebrospinal fluid (7, 86–95). This is reinforced by the recent article by Cramer *et al.* who demonstrated reduced $A\beta$ plaque area and improved cognitive functions in a mouse model of AD after treatment by bexarotene, an agonist of nuclear receptors that transcriptionally regulates ApoE expression (96). Recent and independent genome-wide association studies have identified CLU,

PICALM, CR1, and BIN1 as new markers in the landscape of AD genetic determinants that account for 9, 4, 4, and 4%, respectively, in AD risk across Caucasians (77). Hence, the cause of sporadic or non-Mendelian AD is multifactorial and ApoE alone account only for 20% of the AD risk. This might explain why our results confirm other controversial associations between the variation of plasma ApoE/ApoE4 concentrations and AD. Our results show that neither absolute levels of total ApoE and ApoE4 isoform nor the concentration ratio in heterozygous $\epsilon 3\epsilon 4$ individuals significantly correlated to AD. We addressed this lack of consensus by leveraging the orthogonality of MS-based quantification. Furthermore, we again raise the controversial question of whether ApoE levels are of value in the diagnosis of AD.

* We thank Lyon 1 University, the Institut de Chimie de Lyon and the Centre National de la Recherche Scientifique for their financial assistance in purchasing the 5500 Qtrap instrument. We thank bioMérieux for providing them a temporary access to their laboratories.

☐ This article contains [supplemental Figs. S1 to S4 and Tables S1 to S3](#).

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REFERENCES

- Hatters, D. M., Peters-Libeu, C. A., and Weisgraber, K. H. (2006) Apolipoprotein E structure: insights into function. *Trends Biochem. Sci.* **31**, 445–454
- Verghese, P. B., Castellano, J. M., and Holtzman, D. M. (2011) Apolipoprotein E in Alzheimer's disease and other neurological disorders. *Lancet Neurol.* **10**, 241–252
- Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L., and Pericak-Vance, M. A. (1993) Gene dose of apolipoprotein-E type-4 allele and the risk of Alzheimer's disease in late-onset families. *Science* **261**, 921–923
- Weisgraber, K. H., and Mahley, R. W. (1996) Human apolipoprotein E: The Alzheimer's disease connection. *FASEB J.* **10**, 1485–1494
- Taddei, K., Clarnette, R., Gandy, S. E., and Martins, R. N. (1997) Increased plasma apolipoprotein E (apoE) levels in Alzheimer's disease. *Neurosci. Lett.* **223**, 29–32
- Gupta, V. B., Laws, S. M., Villemagne, V. L., Ames, D., Bush, A. I., Ellis, K. A., Lui, J. K., Masters, C., Rowe, C. C., Szoeki, C., Taddei, K., and Martins, R. N. (2011) Plasma apolipoprotein E and Alzheimer disease risk: The AIBL study of aging. *Neurology* **76**, 1091–1098
- Lehtimäki, T., Pirttilä, T., Mehta, P. D., Wisniewski, H. M., Frey, H., and Nikkari, T. (1995) Apolipoprotein-E (ApoE) polymorphism and its influence on ApoE concentrations in the cerebrospinal-fluid in Finnish patients with Alzheimer's disease. *Human Gen.* **95**, 39–42
- Scacchi, R., Gambina, G., Ruggeri, M., Martini, M. C., Ferrari, G., Silvestri, M., Schiavon, R., and Corbo, R. M. (1999) Plasma levels of apolipoprotein E and genetic markers in elderly patients with Alzheimer's disease. *Neurosci. Lett.* **259**, 33–36
- Slooter, A. J. C., de Knijff, P., Hofman, A., Cruts, M., Breteler, M. M. B., Van Broeckhoven, C., Havekes, L. M., and van Duijn, C. M. (1998) Serum apolipoprotein E level is not increased in Alzheimer's disease: the Rotterdam study. *Neurosci. Lett.* **248**, 21–24
- Solfrizzi, V., Panza, F., Colacicco, A. M., D'Introno, A., Basile, A. M., Capurso, C., Sabba, M., Mascolo, E., and Capurso, A. (2003) Apolipoprotein E (APOE) polymorphism influences serum APOE levels in Alzheimer's disease patients and centenarians. *Int. Psychogeriatrics* **15**, 40–41
- Addona, T. A., Shi, X., Keshishian, H., Mani, D. R., Burgess, M., Gillette, M. A., Clauser, K. R., Shen, D., Lewis, G. D., Farrell, L. A., Fifer, M. A., Sabatine, M. S., Gerszten, R. E., and Carr, S. A. (2011) A pipeline that integrates the discovery and verification of plasma protein biomarkers reveals candidate markers for cardiovascular disease. *Nat. Biotechnol.* **29**, 635–643
- Anderson, L., and Hunter, C. L. (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* **5**, 573–588
- Domon, B., and Aebersold, R. (2010) Options and considerations when selecting a quantitative proteomics strategy. *Nat. Biotechnol.* **28**, 710–721
- Huettenhain, R., Malmstroem, J., Picotti, P., and Aebersold, R. (2009) Perspectives of targeted mass spectrometry for protein biomarker verification. *Curr. Opin. Chem. Biol.* **13**, 518–525
- Lin, S., Shaler, T. A., and Becker, C. H. (2006) Quantification of intermediate-abundance proteins in serum by multiple reaction monitoring mass spectrometry in a single-quadrupole ion trap. *Anal. Chem.* **78**, 5762–5767
- Schiess, R., Wollscheid, B., and Aebersold, R. (2009) Targeted proteomic strategy for clinical biomarker discovery. *Mol. Oncol.* **3**, 33–44
- Surinova, S., Schiess, R., Huettenhain, R., Cerciello, F., Wollscheid, B., and Aebersold, R. (2011) On the development of plasma protein biomarkers. *J. Proteome Res.* **10**, 5–16
- Makawita, S., and Diamandis, E. P. (2010) The bottleneck in the cancer biomarker pipeline and protein quantification through mass spectrometry-based approaches: current strategies for candidate verification. *Clin. Chem.* **56**, 212–222
- Rifai, N., Gillette, M. A., and Carr, S. A. (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol.* **24**, 971–983
- Whiteaker, J. R., Lin, C., Kennedy, J., Hou, L., Trute, M., Sokal, I., Yan, P., Schoenherr, R. M., Zhao, L., Voytovich, U. J., Kelly Spratt, K. S., Krasnoselsky, A., Gafken, P. R., Hogan, J. M., Jones, L. A., Wang, P., Amon, L., Chodosh, L. A., Nelson, P. S., McIntosh, M. W., Kemp, C. J., and Paulovich, A. G. (2011) A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat. Biotechnol.* **29**, 625–U108
- Carr, S. A., and Anderson, L. (2008) Protein quantitation through targeted mass spectrometry: the way out of biomarker purgatory? *Clin. Chem.* **54**, 1749–1752
- Paulovich, A. G., Whiteaker, J. R., Hoofnagle, A. N., and Wang, P. (2008) The interface between biomarker discovery and clinical validation: The tar pit of the protein biomarker pipeline. *Proteomics Clin. Appl.* **2**, 1386–1402
- Hoofnagle, A. N., Becker, J. O., Wener, M. H., and Heinecke, J. W. (2008) Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin. Chem.* **54**, 1796–1804
- Keshishian, H., Addona, T., Burgess, M., Kuhn, E., and Carr, S. A. (2007) Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* **6**, 2212–2229
- Fortin, T., Salvador, A., Charrier, J. P., Lenz, C., Lacoux, X., Morla, A., Choquet-Kastylevsky, G., and Lemoine, J. (2009) Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests. *Mol. Cell. Proteomics* **8**, 1006–1015
- Yang, Y., Qiang, X., Owsiany, K., Zhang, S., Thannhauser, T. W., and Li, L. (2011) Evaluation of different multidimensional LC-MS/MS pipelines for isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis of potato tubers in response to cold storage. *J. Proteome Res.* **10**, 4647–4660
- Kuhn, E., Addona, T., Keshishian, H., Burgess, M., Mani, D. R., Lee, R. T., Sabatine, M. S., Gerszten, R. E., and Carr, S. A. (2009) Developing multiplexed assays for troponin I and interleukin-33 in plasma by peptide immunoaffinity enrichment and targeted mass spectrometry. *Clin. Chem.* **55**, 1108–1117
- Prakash, A., Rezaei, T., Krastins, B., Sarracino, D., Athanas, M., Russo, P., Ross, M. M., Zhang, H., Tian, Y., Kulasingam, V., Drabovich, A. P., Smith, C., Batruch, I., Liotta, L., Petricoin, E., Diamandis, E. P., Chan, D. W., and Lopez, M. F. (2010) Platform for establishing inter laboratory reproducibility of selected reaction monitoring-based mass spectrometry peptide assays. *J. Proteome Res.* **9**, 6678–6688

29. Addona, T. A., Abbatiello, S. E., Schilling, B., Skates, S. J., Mani, D. R., Bunk, D. M., Spiegelman, C. H., Zimmerman, L. J., Ham, A. J. L., Keshishian, H., Hall, S. C., Allen, S., Blackman, R. K., Borchers, C. H., Buck, C., Cardasis, H. L., Cusack, M. P., Dodder, N. G., Gibson, B. W., Held, J. M., Hiltke, T., Jackson, A., Johansen, E. B., Kinsinger, C. R., Li, J., Mesri, M., Neubert, T. A., Niles, R. K., Pulsipher, T. C., Ransohoff, D., Rodriguez, H., Rudnick, P. A., Smith, D., Tabb, D. L., Tegeler, T. J., Varyath, A. M., Vega-Montoto, L. J., Wahlander, A., Waldemarson, S., Wang, M., Whiteaker, J. R., Zhao, L., Anderson, N. L., Fisher, S. J., Liebler, D. C., Paulovich, A. G., Regnier, F. E., Tempst, P., and Carr, S. A. (2009) Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol.* **27**, 633-U85
30. Keshishian, H., Addona, T., Burgess, M., Mani, D. R., Shi, X., Kuhn, E., Sabatine, M. S., Gerszten, R. E., and Carr, S. A. (2009) Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* **8**, 2339–2349
31. Kuhn, E., Whiteaker, J. R., Mani, D. R., Jackson, A. M., Zhao, L., Pope, M. E., Smith, D., Rivera, K. D., Anderson, N. L., Skates, S. J., Pearson, T. W., Paulovich, A. G., and Carr, S. A. (2012) Inter-laboratory evaluation of automated, multiplexed peptide immunoaffinity enrichment coupled to multiple reaction monitoring mass spectrometry for quantifying proteins in plasma. *Mol. Cell. Proteomics* **11**, M111.013854
32. Paulovich, A. G., Billheimer, D., Ham, A. J. L., Vega-Montoto, L., Rudnick, P. A., Tabb, D. L., Wang, P., Blackman, R. K., Bunk, D. M., Cardasis, H. L., Clauser, K. R., Kinsinger, C. R., Schilling, B., Tegeler, T. J., Varyath, A. M., Wang, M., Whiteaker, J. R., Zimmerman, L. J., Fenyo, D., Carr, S. A., Fisher, S. J., Gibson, B. W., Mesri, M., Neubert, T. A., Regnier, F. E., Rodriguez, H., Spiegelman, C., Stein, S. E., Tempst, P., and Liebler, D. C. (2010) Interlaboratory study characterizing a yeast performance standard for benchmarking LC-MS platform performance. *Mol. Cell. Proteomics* **9**, 242–254
33. Selevsek, N., Matondo, M., Sanchez, Carbayo, M., Aebersold, R., and Domon, B. (2011) Systematic quantification of peptides/proteins in urine using selected reaction monitoring. *Proteomics* **11**, 1135–1147
34. Gallien, S., Duriez, E., and Domon, B. (2011) Selected reaction monitoring applied to proteomics. *J. Mass Spectrom.* **46**, 298–312
35. Lange, V., Picotti, P., Domon, B., and Aebersold, R. (2008) Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* **4**, article number 222
36. Pan, S., Aebersold, R., Chen, R., Rush, J., Goodlett, D. R., McIntosh, M. W., Zhang, J., and Brentnall, T. A. (2009) Mass spectrometry based targeted protein quantification: methods and applications. *J. Proteome Res.* **8**, 787–797
37. Shacter, E. (2000) Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* **32**, 307–326
38. Vogt, W. (1995) Oxidation of methionyl residues in proteins - tools, targets, and reversal. *Free Radical Biol. Med.* **18**, 93–105
39. Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6940–6945
40. Huillet, C., Adrait, A., Lebert, D., Picard, G., Trauchessec, M., Louwagie, M., Dupuis, A., Hittinger, L., Ghaleh, B., Le Corvoisier, P., Jaquinod, M., Garin, J., Bruley, C., and Brun, V. (2012) Accurate quantification of cardiovascular biomarkers in serum using Protein Standard Absolute Quantification (PSAQ™) and selected reaction monitoring. *Mol. Cell. Proteomics* **11**, M111.008235
41. Kuzyk, M. A., Smith, D., Yang, J., Cross, T. J., Jackson, A. M., Hardie, D. B., Anderson, N. L., and Borchers, C. H. (2009) Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. Cell. Proteomics* **8**, 1860–1877
42. Proc, J. L., Kuzyk, M. A., Hardie, D. B., Yang, J., Smith, D. S., Jackson, A. M., Parker, C. E., and Borchers, C. H. (2010) A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. *J. Proteome Res.* **9**, 5422–5437
43. Shchepin, R., Moeller, M. N., Kim, H. Y. H., Hatch, D. M., Bartesaghi, S., Kalyanaraman, B., Radi, R., and Porter, N. A. (2010) Tyrosine-lipid peroxide adducts from radical termination: para coupling and intramolecular diels-alder cyclization. *J. Am. Chem. Soc.* **132**, 17490–17500
44. Fukumoto, H., Ingelsson, M., Garevik, N., Wahlund, L. O., Nukina, N., Yaguchi, Y., Shibata, M., Hyman, B. T., Rebeck, G. W., and Irizarry, M. C. (2003) APOE epsilon 3/epsilon 4 heterozygotes have an elevated proportion of apolipoprotein E4 in cerebrospinal fluid relative to plasma, independent of Alzheimer's disease diagnosis. *Exp. Neurol.* **183**, 249–253
45. Wildsmith, K. R., Han, B., and Bateman, R. J. (2009) Method for the simultaneous quantitation of apolipoprotein E isoforms using tandem mass spectrometry. *Anal. Biochem.* **395**, 116–118
46. Agger, S. A., Marney, L. C., and Hoofnagle, A. N. (2010) Simultaneous quantification of apolipoprotein A-I and apolipoprotein B by liquid-chromatography-multiple-reaction-monitoring mass spectrometry. *Clin. Chem.* **56**, 1804–1813
47. Ghesquiere, B., Jonckheere, V., Colaert, N., Van Durme, J., Timmerman, E., Goethals, M., Schymkowitz, J., Rousseau, F., Vandekerckhove, J., and Gevaert, K. (2011) Redox proteomics of protein-bound methionine oxidation. *Mol. Cell. Proteomics* **10**, M110.006866
48. Gevaert, K., Pinxteren, J., Demol, H., Hugelier, K., Staes, A., Van Damme, J., Martens, L., and Vandekerckhove, J. (2006) Four stage liquid chromatographic selection of methionyl peptides for peptide-centric proteome analysis: The proteome of human multipotent adult progenitor cells. *J. Proteome Res.* **5**, 1415–1428
49. Gevaert, K., Van Damme, J., Goethals, M., Thomas, G. R., Hoorelbeke, B., Demol, H., Martens, L., Puype, M., Staes, A., and Vandekerckhove, J. (2002) Chromatographic isolation of methionine-containing peptides for gel-free proteome analysis - Identification of more than 800 Escherichia coli proteins. *Mol. Cell. Proteomics* **1**, 896–903
50. Hoofnagle, A. N., Becker, J. O., Oda, M. N., Cavigliolo, G., Mayer, P., and Vaisar, T. (2012) Multiple-reaction monitoring-mass spectrometric assays can accurately measure the relative protein abundance in complex mixtures. *Clin. Chem.* **58**, 777–781
51. Lassman, M. E., McLaughlin, T. M., Somers, E. P., Stefanni, A. C., Chen, Z., Murphy, B. A., Bierilo, K. K., Flattery, A. M., Wong, K. K., Castro-Perez, J. M., Hubbard, B. K., and Roddy, T. P. (2012) A rapid method for cross-species quantitation of apolipoproteins A1, B48 and B100 in plasma by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **26**, 101–108
52. Percy, A. J., Chambers, A. G., Yang, J., Domanski, D., and Borchers, C. H. (2012) Comparison of standard- and nano-flow liquid chromatography platforms for MRM-based quantitation of putative plasma biomarker proteins. *Anal. Bioanal. Chem.* DOI 10.1007/s00216-012-6010
53. Trenchevska, O., Kamcheva, E., and Nedelkov, D. (2011) Mass spectrometric immunoassay for quantitative determination of transthyretin and its variants. *Proteomics* **11**, 3633–3641
54. Anderson, N. L., Anderson, N. G., Haines, L. R., Hardie, D. B., Olafson, R. W., and Pearson, T. W. (2004) Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). *J. Proteome Res.* **3**, 235–244
55. Whiteaker, J. R., Zhao, L., Abbatiello, S. E., Burgess, M., Kuhn, E., Lin, C., Pope, M. E., Razavi, M., Anderson, N. L., Pearson, T. W., Carr, S. A., and Paulovich, A. G. (2011) Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. *Mol. Cell. Proteomics* **10**, M110.005645
56. Beekman, M., Heijmans, B. T., Martin, N. G., Pedersen, N. L., Whitfield, J. B., DeFaire, U., van Baal, G. C. M., Snieder, H., Vogler, G. P., Slagboom, P. E., and Boomsma, D. I. (2002) Heritabilities of apolipoprotein and lipid levels in three countries. *Twin Res.* **5**, 87–97
57. Riddell, D. R., Zhou, H., Atchison, K., Warwick, H. K., Atkinson, P. J., Jefferson, J., Xu, L., Aschmies, S., Kirksey, Y., Hu, Y., Wagner, E., Parratt, A., Xu, J., Li, Z., Zaleska, M. M., Jacobsen, J. S., Pangalos, M. N., and Reinhart, P. H. (2008) Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. *J. Neurosci.* **28**, 11445–11453
58. Beekman, M., Posthuma, D., Heijmans, B. T., Lakenberg, N., Suchiman, H. E. D., Snieder, H., de Knijff, P., Frants, R. R., van Ommen, G. J. B., Kluff, C., Vogler, G. P., Slagboom, P. E., and Boomsma, D. I. (2004) Combined association and linkage analysis applied to the APOE locus. *Genetic Epidemiol.* **26**, 328–337
59. Uchida, Y., Ito, S., and Nukina, N. (2000) Sandwich ELISA for the measurement of Apo-E4 levels in serum and the estimation of the allelic status of Apo-E4 isoforms. *J. Clin. Lab. Anal.* **14**, 260–264
60. Bohnet, K., Pillot, T., Visvikis, S., Sabolovic, N., and Siest, G. (1996) Apolipoprotein (apo) E genotype and apoE concentration determine

- binding of normal very low density lipoproteins to HepG2 cell surface receptors. *J. Lipid Res.* **37**, 1316–1324
61. Brun, V., Dupuis, A., Adrait, A., Marcellin, M., Thomas, D., Court, M., Vandenesch, F., and Garin, J. (2007) Isotope-labeled protein standards. *Mol. Cell. Proteomics* **6**, 2139–2149
 62. Haddy, N., De Bacquer, D., Chemaly, M. M., Maurice, M., Ehnholm, C., Evans, A., Sans, S., Martins, M. D., De Backer, G., Siest, G., and Visvikis, S. (2002) The importance of plasma concentration in addition apolipoprotein E to its common polymorphism on inter-individual variation in lipid levels: results from Apo Europe. *Eur. J. Human Gen.* **10**, 841–850
 63. Schiele, F., De Bacquer, D., Vincent-Viry, M., Beisiegel, U., Ehnholm, C., Evans, A., Kafatos, A., Martins, M. C., Sans, S., Sass, C., Visvikis, S., De Backer, G., and Siest, G. (2000) Apolipoprotein E serum concentration and polymorphism in six European countries: the ApoEurope Project. *Atherosclerosis* **152**, 475–488
 64. Artiga, M. J., Bullido, M. J., Sastre, I., Recuero, M., Garcia, M. A., Aldudo, J., Vazquez, J., and Valdivieso, F. (1998) Allelic polymorphisms in the transcriptional regulatory region of apolipoprotein E gene. *Febs Letts.* **421**, 105–108
 65. Bekris, L. M., Millard, S. P., Galloway, N. M., Vuletic, S., Albers, J. J., Lie, G., Galasko, D. R., DeCarli, C., Farlow, M. R., Clark, C. M., Quinn, J. F., Kaye, J. A., Schellenberg, G. D., Tsuang, D., Peskind, E. R., and Yu, C. E. Multiple SN (2008) Ps within and surrounding the apolipoprotein E gene influence cerebrospinal fluid apolipoprotein E protein levels. *J. Alzheimers Dis.* **13**, 255–266
 66. Lambert, J. C., Berr, C., Pasquier, F., Delacourte, A., Frigard, B., Cottel, D., Perez-Tur, J., Mouroux, V., Mohr, M., Cecyry, D., Galasko, D., Lendon, C., Poirier, J., Hardy, J., Mann, D., Amouyel, P., and Chartier-Harlin, M. C. (1998) Pronounced impact of Th1/E47cs mutation compared with -491 AT mutation on neural APOE gene expression and risk of developing Alzheimer's disease. *Human Mol. Gen.* **7**, 1511–1516
 67. Lambert, J. C., Brousseau, T., Defosse, V., Evans, A., Arveiler, D., Ruidavets, J. B., Haas, B., Cambou, J. P., Luc, G., Ducimetiere, P., Cambien, F., Chartier-Harlin, M. C., and Amouyel, P. (2000) Independent association of an APOE gene promoter polymorphism with increased risk of myocardial infarction and decreased APOE plasma concentrations - the ECTIM Study. *Human Mol. Gen.* **9**, 57–61
 68. Laws, S. M., Hone, E., Taddei, K., Harper, C., Dean, B., McClean, C., Masters, C., Lautenschlager, N., Gandy, S. E., and Martins, R. N. (2002) Variation at the APOE-491 promoter locus is associated with altered brain levels of apolipoprotein E. *Mol. Psychiatry* **7**, 886–890
 69. Laws, S. M., Taddei, K., Martins, G., Paton, A., Fisher, C., Clarnette, R., Hallmayer, J., Brooks, W. S., Gandy, S. E., and Martins, R. N. (1999) The -491AA polymorphism in the APOE gene is associated with increased plasma apoE levels in Alzheimer's disease. *Neuroreport* **10**, 879–882
 70. Bullido, M. J., Artiga, M. J., Recuero, M., Sastre, I., Garcia, M. A., Aldudo, J., Lendon, C., Han, S. W., Morris, J. C., Frank, A., Vazquez, J., Goate, A., and Valdivieso, F. (1998) A polymorphism in the regulatory region of APOE associated with risk for Alzheimer's dementia. *Nat. Gen.* **18**, 69–71
 71. Parker, G. R., Cathcart, H. M., Huang, R., Lanham, I. S., Corder, E. H., and Poduslo, S. E. (2005) Apolipoprotein gene E4 allele promoter polymorphisms as risk factors for Alzheimer's disease. *Psychiatric Gen.* **15**, 271–275
 72. Ramos, M. C., Matias, S., Artiga, M. J., Pozueta, J., Sastre, I., Valdivieso, F., and Bullido, M. J. (2005) Neuronal specific regulatory elements in apolipoprotein E gene proximal promoter. *Neuroreport* **16**, 1027–1030
 73. Town, T., Paris, D., Fallin, D., Barker, R., Duara, R., Gold, M., Crawford, F., and Mullan, M. (1998) The -491A/T apolipoprotein E promoter polymorphism association with Alzheimer's disease: independent risk and linkage disequilibrium with the known APOE polymorphism. *Neurosci. Lett.* **252**, 95–98
 74. Wang, J. C., Kwon, J. M., Shah, P., Morris, J. C., and Goate, A. (2000) Effect of APOE genotype and promoter polymorphism on risk of Alzheimer's disease. *Neurology* **55**, 1644–1649
 75. Yu, C. E., Seltman, H., Peskind, E. R., Galloway, N., Zhou, P. X., Rosenthal, E., Wijsman, E. M., Tsuang, D. W., Devlin, B., and Schellenberg, G. D. (2007) Comprehensive analysis of APOE and selected proximate markers for late-onset Alzheimer's disease: Patterns of linkage disequilibrium and disease/marker association. *Genomics* **89**, 655–665
 76. Lambert, J. C., and Amouyel, P. (2007) Genetic heterogeneity of Alzheimer's disease: Complexity and advances. *Psychoneuroendocrinology* **32**, S62–S70
 77. Lambert, J. C., and Amouyel, P. (2011) Genetics of Alzheimer's disease: new evidences for an old hypothesis? *Curr. Opinion Gen. Develop.* **21**, 295–301
 78. Bales, K. R., Liu, F., Wu, S., Lin, S., Koger, D., DeLong, C., Hansen, J. C., Sullivan, P. M., and Paul, S. M. (2009) Human APOE isoform-dependent effects on brain beta-amyloid levels in PDAPP transgenic mice. *J. Neurosci.* **29**, 6771–6779
 79. Fryer, J. D., Simmons, K., Parsadanian, M., Bales, K. R., Paul, S. M., Sullivan, P. M., and Holtzman, D. M. (2005) Human apolipoprotein E4 alters the amyloid-beta 40 : 42 ratio and promotes the formation of cerebral amyloid angiopathy in an amyloid precursor protein transgenic model. *J. Neurosci.* **25**, 2803–2810
 80. Fagan, A. M., Watson, M., Parsadanian, M., Bales, K. R., Paul, S. M., and Holtzman, D. M. (2002) Human and murine ApoE markedly alters A beta metabolism before and after plaque formation in a mouse model of Alzheimer's disease. *Neurobiol. Disease* **9**, 305–318
 81. Holtzman, D. M., Bales, K. R., Tenkova, T., Fagan, A. M., Parsadanian, M., Sartorius, L. J., Mackey, B., Olney, J., McKeel, D., Wozniak, D., and Paul, S. M. (2000) Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2892–2897
 82. Chiang, G. C., Insel, P. S., Tosun, D., Schuff, N., Truran-Sacrey, D., Raptentsetsang, S. T., Jack, C. R., Jr., Aisen, P. S., Petersen, R. C., and Weiner, M. W. (2010) Hippocampal atrophy rates and CSF biomarkers in elderly APOE2 normal subjects. *Neurology* **75**, 1976–1981
 83. Morris, J. C., Roe, C. M., Xiong, C., Fagan, A. M., Goate, A. M., Holtzman, D. M., and Mintun, M. A. (2010) APOE Predicts Amyloid-Beta but Not Tau Alzheimer Pathology in Cognitively Normal Aging. *Ann. Neurol.* **67**, 122–131
 84. Reiman, E. M., Chen, K., Liu, X., Bandy, D., Yu, M., Lee, W., Ayutyanont, N., Keppler, J., Reeder, S. A., Langbaum, J. B. S., Alexander, G. E., Klunk, W. E., Mathis, C. A., Price, J. C., Aizenstein, H. J., DeKosky, S. T., and Caselli, R. J. (2009) Fibrillar amyloid-beta burden in cognitively normal people at 3 levels of genetic risk for Alzheimer's disease. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 6820–6825
 85. Sunderland, T., Mirza, N., Putnam, K. T., Linker, G., Bhupali, D., Durham, R., Soares, H., Kimmel, L., Friedman, D., Bergeson, J., Csako, G., Levy, J. A., Bartko, J. J., and Cohen, R. M.: (2004) Cerebrospinal fluid beta-amyloid(1–42) and tau in control subjects at risk for Alzheimer's disease: The effect of APOE epsilon 4 allele. *Biol. Psychiatry* **56**, 670–676
 86. Blennow, K., Hesse, C., and Fredman, P. (1994) Cerebrospinal-fluid apolipoprotein-E is reduced in Alzheimers-disease. *Neuroreport* **5**, 2534–2536
 87. Carlsson, J., Armstrong, V. W., Reiber, H., Felgenhauer, K., and Seidel, D. (1991) Clinical Relevance of the Quantification of Apolipoprotein-E in Cerebrospinal-Fluid. *Clin. Chim. Acta* **196**, 167–176
 88. Fukuyama, R., Mizuno, T., Mori, S., Yanagisawa, K., Nakajima, K., and Fushiki, S. (2000) Age-dependent decline in the apolipoprotein e level in cerebrospinal fluid from control subjects and its increase in cerebrospinal fluid from patients with Alzheimer's disease. *Eur. Neurol.* **43**, 161–169
 89. Hesse, C., Larsson, H., Fredman, P., Minthon, L., Andreasson, N., Davidsson, P., and Blennow, K. (2000) Measurement of apolipoprotein E (apoE) in cerebrospinal fluid. *Neurochem. Res.* **25**, 511–517
 90. Kunicki, S., Richardson, J., Mehta, P. D., Kim, K. S., and Zorychta, E. (1998) The effects of age, apolipoprotein E phenotype and gender on the concentration of amyloid-beta (A beta) 40, A beta 42, apolipoprotein E and transthyretin in human cerebrospinal fluid. *Clin. Biochem.* **31**, 409–415
 91. Landen, M., Hesse, C., Fredman, P., Regland, B., Wallin, A., and Blennow, K. (1996) Apolipoprotein E in cerebrospinal fluid from patients with Alzheimer's disease and other forms of dementia is reduced but without any correlation to the apoE4 isoform. *Dementia* **7**, 273–278
 92. Lefranc, D., Vermersch, P., Dallongeville, J., DaemsMonpeurt, C., Petit, H., and Delacourte, A. (1996) Relevance of the quantification of apolipoprotein E in the cerebrospinal fluid in Alzheimer's disease. *Neurosci. Letts.* **212**, 91–94
 93. Rosler, N., Wichart, I., Bancher, C., and Jellinger, K. A. (1996) Tau protein and apolipoprotein E in CSF diagnostics of Alzheimer's disease: Impact on non-Alzheimer's dementia? *J. Neural Trans. Supplement* **49**, 259–266

94. Skoog, I., Hesser, C., Fredman, P., Andreasson, L. A., Palmertz, B., and Blennow, K. (1997) Apolipoprotein E in cerebrospinal fluid in 85-year-old subjects - Relation to dementia, apolipoprotein E polymorphism, cerebral atrophy, and white matter lesions. *Arch. Neurol.* **54**, 267–272
95. Song, H., Saito, K., Seishima, M., Noma, A., Urakami, K., and Nakashima, K. (1997) Cerebrospinal fluid apo E and apo A-I concentrations in early and late-onset Alzheimer's disease. *Neurosci. Letts.* **231**, 175–178
96. Cramer, P. E., Cirrito, J. R., Wesson, D. W., Lee, C. Y. D., Karlo, J. C., Zinn, A. E., Casali, B. T., Restivo, J. L., Goebel, W. D., James, M. J., Brunden, K. R., Wilson, D. A., and Landreth, G. E. (2012) ApoE-Directed Therapeutics Rapidly Clear beta-Amyloid and Reverse Deficits in AD Mouse Models. *Science* **335**, 1503–1506