Automated Multiplex LC-MS/MS Assay for Quantifying Serum Apolipoproteins A-I, B, C-I, C-II, C-III, and E with Qualitative Apolipoprotein E Phenotyping

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BACKGROUND: Direct and calculated measures of lipoprotein fractions for cardiovascular risk assessment suffer from analytical inaccuracy in certain dyslipidemic and pathological states, most commonly hypertriglyceridemia. LC-MS/MS has proven suitable for multiplexed quantification and phenotyping of apolipoproteins. We developed and provisionally validated an automated assay for quantification of apolipoprotein (apo) A-I, B, C-I, C-II, C-III, and E and simultaneous qualitative assessment of apoE phenotypes.

METHODS: We used 5 value-assigned human serum pools for external calibration. Serum proteins were denatured, reduced, and alkylated according to standard mass spectrometry–based proteomics procedures. After trypsin digestion, peptides were analyzed by LC-MS/MS. For each peptide, we measured 2 transitions. We compared LC-MS/MS results to those obtained by an immunoturbidimetric assay. We used 5 value-assigned human serum pools for external calibration. Serum proteins were denatured, reduced, and alkylated according to standard mass spectrometry–based proteomics procedures. After trypsin digestion, peptides were analyzed by LC-MS/MS. For each peptide, we measured 2 transitions. We compared LC-MS/MS results to those obtained by an immunoturbidimetric assay.

RESULTS: Intraassay CVs were 2.3%–5.5%, and total CVs were 2.5%–5.9%. The LC-MS/MS assay correlated (R = 0.975–0.999) with immunoturbidimetric assays with Conformité Européenne marking for apoA-I, apoB, apoC-II, apoC-III, and apoE in normotriglyceridemic (n = 54) and hypertriglyceridemic (n = 46) sera. Results were interchangeable for apoA-I ≤3.0 g/L (Deming slope 1.014) and for apoB-100 ≤1.8 g/L (Deming slope 1.016) and were traceable to higher-order standards.

CONCLUSIONS: The multiplex format provides an opportunity for new diagnostic and pathophysiological insights into types of dyslipidemia and allows a more personalized approach for diagnosis and treatment of lipid abnormalities.

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available, which impedes discovery of pathophysiological clues (e.g., apoC-II deficiencies or apoC-III elevations) for correct clinical diagnosis and adequate treatment of dyslipidemia.

LC-MS/MS is a powerful approach for highly multiplexed protein quantification and isoform differentiation (11–14) and could support clinical studies to elucidate the role of apolipoproteins and their variants in CVD as well as the implementation of apolipoprotein measurements in clinical practice. Various LC-MS/MS assays for multiplexed quantification of apolipoproteins (14–23) or phenotyping of apoE (24–27) have been reported. However, the clinical applicability and throughput of these assays are often limited by the complexity and processing time of the manual sample preparation that is typically performed. Clinical application of LC-MS/MS for protein biomarkers, furthermore, requires stringent criteria for bias and precision (28, 29), which are particularly challenging in workflows involving enzymatic digestion of the protein measurand before LC-MS/MS peptide measurement (30).

Here, we describe the development of an LC-MS/MS method for multiplexed quantification of serum apoA-I, apoB, apoC-I, apoC-II, apoC-III, and apoE, including various validation steps toward clinical implementation. In addition, this mass spectrometry (MS)–based strategy allows qualitative phenotyping of apoE, which is currently based on genotyping or gel-based phenotyping. To establish metrological traceability, our laboratory-developed test has been extensively characterized with respect to analytical quality requirements for quantitative proteomics assays (29); the effects of trypsin digestion on peptide selection, formation, and degradation; and evaluation of accuracy and precision (19, 29–31). We previously demonstrated that manual sample preparation and matrix effects are major sources of error (30). In the current study, we report on the optimization and implementation of sample preparation with a liquid handling pipetting robot, as well as the accuracy and robustness of the multiplexed LC-MS/MS assay.

Materials and Methods

All solvents were LC-MS/MS grade, and reagents used were of the highest available purity. Sample preparations and instrumental conditions, as well as freeze-thaw and temperature stability data on the serum samples, are detailed in Supplemental Data, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue1.

SERA AND CALIBRATION

We obtained 5 normotriglyceridemic (NTG) [triglycerides ≤2.3 mmol/L (204 mg/dL)] sera (NTG1, NTG2, NTG3, NTG4, and NTG5) and 3 hypertriglyceridemic (HTG) (triglycerides >2.3 mmol/L) value-assigned sera (HTG1, HTG2, and HTG3), prepared according to CLSI C37-A, from the Dutch External Quality Assessment organization (Nijmegen, the Netherlands). We collected patient sera with cholesterol concentrations <10 mmol/L and either healthy (<2.3 mmol/L; n = 54) or high [2.3–20 mmol/L (204–1770 mg/dL); n = 46] triglyceride concentrations from fully anonymized patient blood samples previously processed for analysis in our department. In addition, 2 serum pools were created with healthy triglyceride and cholesterol concentrations (NTG6 and NTG7). All patient sera were collected in a gel-containing Serum Separator Vacutainer Tube (BD Diagnostics), centrifuged at 1000 g for 10 min, and stored at −80 °C in single-use aliquots. Triglyceride and cholesterol compositions (except those for NTG6 and NTG7) can be found elsewhere (30).

INSTRUMENTATION

Samples were prepared on a Bravo 96-channel liquid handling platform (Agilent Technologies) allowing semi-automated processing. We performed LC-MS/MS analysis on an Agilent 1290 UHPLC system coupled to an Agilent 6490 triple-quadrupole mass spectrometer operated in positive-ion multiple reaction monitoring (MRM) mode. A representative LC-MS/MS chromatogram is presented in online Supplemental Fig. 1. MRM transitions for all peptides are listed in online Supplemental Table 1.

SIGNATURE PEPTIDE SELECTION

We selected 2 signature peptides per apolipoprotein on the basis of our previous work, published literature (17–19, 30), and signal intensities during LC-MS/MS analyses of tryptic peptide candidates. The peptide with the most reproducible results was selected as quantification peptide, whereas the other peptide was used for confirmation (23). In addition, a third differential peptide was selected for apoB and apoE to allow differentiation between apoB-100 and total apoB (i.e., the sum of apoB-100 and apoB-48), or between total apoE and non-apoE2. For phenotyping of apoE2, apoE3, and apoE4, we also included 3 phenotyping peptides (25–27). All quantification, differential, and phenotyping peptides are listed in Table 1. We used stable isotope–labeled (SIL) peptides as internal standards (see online Supplemental Data for details).

CALIBRATION AND TRACEABILITY

We used 5 native serum samples (NTG1, NTG2, NTG3, NTG4, and NTG5), prepared in triplicate and distributed throughout each run, for daily calibration. The sera were value-assigned for apoA-I and apoB by the Northwest Lipid Metabolism and Diabetes Research Laboratories (Seattle, WA) and were traceable to WHO.
international reference material SP1–01 and SP3–07, respectively (32, 33). ApoC-II, apoC-III, and apoE concentrations were value-assigned in-house by immunoturbidimetric assay (ITA) with a Modular P800 analyzer (Roche Diagnostics) and product calibrators with Conformité Européenne marking (Randox Laboratories). Concentrations of apoC-I were assigned with ELISA (Assaypro). The traceability of the 6 apolipoproteins is depicted in Fig. 1. For construction of daily calibration curves and quantification of apolipoprotein concentrations, we used least square regression on the basis of 3 replicate preparations of the 5 calibration samples. Because ELISA results for apoC-I correlated poorly with the LC-MS/MS response and had a narrow concentration range (18.9–26.2 mg/L), 1-point calibration was performed for apoC-I (on the basis of NTG4). Value-assigned concentrations are listed in online Supplemental Table 2.

METHOD VALIDATION

We evaluated intraassay, interassay, and total precision by quadruplicate sample preparations of 5 samples (HTG1, HTG2, HTG3, NTG6, and NTG7) on 5 different days according to CLSI protocol EP15-A2 (34). In addition, we analyzed pooled serum digests, aliquoted and stored at –80 °C, in quadruplicate at the beginning and the end of each run to act as QC for LC-MS/MS performance. Quantification of apoA-I, apoB, apoC-II, apoC-III, and apoE by LC-MS/MS was compared with ITA for 100 patient samples according to CLSI EP09-A3 (35). Linearity of the LC-MRM-MS method was evaluated according to CLSI EP-06 (36) by generation of dilution curves from calibrator (NTG) samples diluted with 4% human serum albumin (HSA) in saline (Albuman® containing 40 g/L albumin, Sanquin) and from patient samples with high apo concentrations diluted with human serum with low apo concentrations. All dilution curves were prepared in triplicate and included 0:1, 1:3, 1:1, 3:1, 7:1, and 1:0 vol/vol mixtures of diluent and starting matrix. Detailed specifications about all method validation procedures are described in online Supplemental Data.

Results

CALIBRATION

Mean calibration results for the quantification peptides of the 6 apolipoproteins are summarized in online Supplemental Table 2. For all quantification peptides, the mean determination coefficient ($R^2$) was 0.960–0.981. All slopes of all peptides varied over 5 different days, including the slopes of the peptides from apoC-I on the basis of a single-point calibration. The calibration curve for the differential apoE2 peptide $\text{LAVYQAGAR}$ resulted in an $R^2$ of 0.891. In addition, there was a consistent negative deviation for the MS response of apoA-I and apoB in sample NTG5, in concordance with previous observations as well as ITA measurements (30).

PRECISION EVALUATION

Intraassay CVs were 2.3%–5.5% for all quantification and differential peptides, whereas the mean total CV, calculated over the 5 different samples, was 2.5%–5.9%.

<table>
<thead>
<tr>
<th>Table 1. Signature peptides used for apo quantification and apoE phenotyping.a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td>Quantification peptides (SIL analog included)</td>
</tr>
<tr>
<td>ApoA-I</td>
</tr>
<tr>
<td>ApoB</td>
</tr>
<tr>
<td>ApoC-I</td>
</tr>
<tr>
<td>ApoC-II</td>
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<tr>
<td>ApoC-III</td>
</tr>
<tr>
<td>ApoE</td>
</tr>
<tr>
<td>Differential peptides (SIL analog included)</td>
</tr>
<tr>
<td>ApoB-100</td>
</tr>
<tr>
<td>Non-apoE2</td>
</tr>
<tr>
<td>Phenotyping peptides (no SIL analog included)</td>
</tr>
<tr>
<td>ApoE2</td>
</tr>
<tr>
<td>Non-apoE4</td>
</tr>
<tr>
<td>ApoE4</td>
</tr>
</tbody>
</table>

a $\text{C}_{\text{CM}}$ denotes the measurement of a carbamyldimethylated cysteine.
**Fig. 1.** Scheme to transfer trueness for LC-MS/MS quantification of apoA-I and apoB to the primary reference standard (left), and for apoC-I, apoC-II, apoC-III, and apoE (right) to product calibrators provided by the manufacturer.

The protein measurands are represented by specific signature peptides in the LC-MS/MS assay (protein amino acid positions are defined in brackets). NLMDR, Northwest Lipid Metabolism and Diabetes Research Laboratories.
Table 2. Intra-assay and total CVs for multiplexed quantification of apolipoproteins by LC-MS/MS.\(^a\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Intra-assay CV, %</th>
<th>Total CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantification peptides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I</td>
<td>VQPYLDDFQK</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>ApoB</td>
<td>TEVIPPLIENR</td>
<td>2.3</td>
<td>2.5</td>
</tr>
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<td>ApoC-I</td>
<td>TPDVSSALDK</td>
<td>5.5</td>
<td>5.9</td>
</tr>
<tr>
<td>ApoC-II</td>
<td>TLYPAVDEK</td>
<td>5.4</td>
<td>5.8</td>
</tr>
<tr>
<td>ApoC-III</td>
<td>GWVTDGFSSLK</td>
<td>4.4</td>
<td>4.5</td>
</tr>
<tr>
<td>ApoE</td>
<td>SEELQQFVAAEET</td>
<td>3.2</td>
<td>3.9</td>
</tr>
<tr>
<td>Differential peptides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoB-100</td>
<td>FPEVDVLTK</td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Non-apoE2</td>
<td>LAVYYQAGAR</td>
<td>5.2</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* Mean of 5 different samples; all samples were prepared in quadruplicate on 5 different days, using daily calibration for quantification (see online Supplemental Table 3).

Quantification of Apolipoproteins by LC-MS/MS

Detailed results per sample type for both quantification and confirmation peptides are presented in online Supplemental Table 3. The intraassay CV of LC-MS/MS measurement (i.e., on the basis of pooled serum digests and, hence, without variability from sample preparation and digestion) was 1.4%–4.5% for the same quantification and differential peptides.

**METHOD COMPARISON**

Scatterplots and Deming regression results for comparison between quantification of serum apoA-I, apoB, apoB-100, apoC-II, apoC-III, and apoE with LC-MS/MS and ITA are presented in Fig. 2 (EValuator\(^b\)) (see online Supplemental Data). LC-MS/MS and ITA results correlated well (\(R = 0.974–0.995\)). Furthermore, the LC-MS/MS and ITA results for apoA-I and apoB were interchangeable within a quantitative range \(\leq 3.0\) g/L for apoA-I and \(\leq 1.8\) g/L for apoB (Deming slopes of 1.014 and 1.016, respectively). Of note, the results of the LC-MS/MS assay for apoB were interchangeable with ITA results only when LC-MS/MS quantification was based on the apoB-100–specific differential peptide **FPEVDVLTK**. On the other hand, LC-MS/MS quantification of total apoB on the basis of the quantification peptide **TEVIPPLIENR** resulted in a slightly positive bias compared with ITA (Deming slope 1.097). Likewise, results for apoC-II, apoC-III, and total apoE were higher with LC-MS/MS than with ITA (Deming slopes 1.241, 1.084, and 1.118, respectively). No appropriate clinical test was available to compare the LC-MS/MS results for apoC-I. Correlation and agreement between LC-MS/MS and ELISA (with known interference from apoB) were poor (\(R = 0.744\) and Deming slope 2.053), as illustrated in online Supplemental Fig. 2.

**PEPTIDE COMPARISON**

The results on the basis of the quantification and confirmation peptides for quantification of total apoB agreed well (Deming slope 1.006), whereas the apoB-100–specific differential peptide **FPEVDVLTK** generally provided slightly lower concentrations (Deming slope 0.950) than the quantification peptide for total apoB (Fig. 3). Quantification of total apoE resulted in lower concentrations (<20%) with the quantification peptide compared with the confirmation peptide in 3 samples. In 2 samples, the same disagreement was observed in comparison to the non-apoE2–specific peptide, whereas for the other sample, the concentration of non-apoE2 was lower than the total apoE concentration on the basis of either quantification or confirmation peptide. All negative deviations for non-apoE2 (\(n = 12\)) were confirmed by detection of the apoE2-specific peptide **CLAVYQAAGAR** (Fig. 3). Comparison of apoA-I concentrations showed only 1 sample with a deviation outside 20% between the 2 peptides. Concentrations of apoC-II and apoC-III agreed well between quantification and confirmation peptides (Deming slopes 0.986 and 1.012, respectively). Comparison of the apoC-I concentrations showed lower concentrations on the basis of the quantification peptide **TPDVSSALDK** than the confirmation peptides **EFGNTLEDK** in several samples in the lower concentration range (Fig. 3).

**LINEARITY**

The linearity of the LC-MS/MS responses is illustrated in Fig. 4 for all quantification peptides. Dilution of the calibration samples with 4% HSA in saline resulted in excellent linearity for all peptides from apoA-I (0–2.2 g/L), total apoB (0–1.3 g/L), apoC-II (0–56 mg/L), apoC-III (0–129 mg/L), and total apoE (0–33 mg/L).
Although the sera were not specifically assigned for apoB-100, apoC-I, or non-apoE2, responses were also within the 10% allowable nonlinearity for the 5 highest concentration values of these apolipoproteins. In addition, dilution of sera with apo concentrations outside the calibration range with a patient serum with low apo concentration resulted in upper limits of linearity of 3.2 g/L for apoA-I, 110 mg/L for apoC-II, 287 mg/L for apoC-III, and 100 mg/L for apoE (see online Supplemental Data). Dilution of hypertriglyceridemic serum [17.3 mmol/L (1531 mg/dL) triglycerides] resulted in linear responses for all peptides from apoA-I, apoB, and apoE, as well as the confirmation peptides from apoC-I and apoC-II, indicating that the triglyceride contents in these sera do not affect sample preparation, digestion efficiency, or LC-MS/MS performance for these peptides. On the other hand, for apoC-III and the quantification peptides from apoC-I and apoC-II, deviation outside the 10% allowable nonlinearity was observed in samples (Fig. 4). Whether this nonlinearity can be attributed to the triglyceride content cannot be concluded, since the apo concentrations in these samples were outside the demonstrated linear range in normotriglyceridemic sera.

**ApoE PHENOTYPING**

The results for apoE phenotypes in the 100 clinical sera are presented in online Supplemental Fig. 4. The differential peptide for non-apoE2 was identified (and quantified) in all sera, and the presence of the apoE2/2 pheno-
type could therefore be excluded in this sample cohort (signal intensities and thresholds are detailed in online Supplemental Data). The apoE2/4 phenotype was identified in 4 patients, and the apoE2/3 phenotype, in 11 patients. For 1 NTG sample (total apoE concentration 47 mg/L), the presence of the apoE2-specific peptide CLAVYGAR could be neither demonstrated nor unambiguously excluded. Interestingly, for 3 of the 4 patients with apoE2/4 phenotype, the concentration of non-apoE2 was >80% of the total apoE concentration, indicating a low concentration of apoE2 relative to apoE4. In contrast, the concentration of non-apoE2 in the patients with apoE2/3 phenotype was 25%–50% of the total apoE concentration in 10 of the 11 samples, indicating a relatively high concentration of apoE2 compared with apoE3. Besides the 4 patients with apoE2/4 phenotype, the apoE4-specific peptide LGADMEDVR was detected in 29 patients with apoE3/4 phenotype and 4 patients with apoE4/4 phenotype. For 3 samples, however, the presence of the apoE4-specific peptide LGADMEDVR could be neither demonstrated nor unambiguously excluded, suggesting either apoE3/3 or apoE3/4 phenotype. Exactly 50% of the identified samples presented the most common apoE3/3 phenotype. Finally, it should be noted that for these 100 clinical serum samples, apoE genotyping or gel-based phenotyping (i.e., orthogonal confirmation) was not performed.

**Discussion**

CVD is a major cause of morbidity and mortality. Accordingly, reduction of CVD burden is a priority area for health care providers in general practice, internal medicine, cardiology, endocrinology, and lipidology. Clinical practice guidelines on managing dyslipidemia and reducing cardiovascular risk are widely available and regularly revised by various national and international professional organizations. The majority of clinical laboratories provide lipid results for diagnostic, prognostic, and monitoring purposes, limited to total cholesterol, triglycerides, direct or indirect HDL cholesterol determination, direct or calculated LDL cholesterol determination, and non-HDL calculation. All of these tests suffer from analytical deficiencies (i.e., nonselectivity) in specific pathophysiological states (10). Although some routine laboratories also offer measurement of apoA-I and apoB-100, more
sophisticated testing than this is limited to the reference laboratory setting. The present study offers potential expansion of the apolipoprotein panel to 6 quantities in 1 workflow, with the benefit of simultaneous apoE phenotyping.

Our previously reported manual sample preparation procedure for serum apoA-I and apoB (19, 30, 31) was optimized to improve peptide recoveries and digestion rates for all apolipoproteins (see online Supplemental Data and online Supplemental Fig. 6). In addition, automation of all liquid-handling steps improved robustness and allowed quantification of apoA-I and apoB within the minimal or desirable requirements for precision on the basis of biological variation (e.g., minimal CV for apoA-I <5% and desirable CV for apoB <3.5%). Quantification of the other, lower-abundance apolipoproteins was within 5.9% total precision and outperformed our identical precision evaluation with the previous manual protocol (data not shown), underlining the importance of automation in workflows like the one we describe.

Method comparison demonstrated that results of the LC-MS/MS assay and ITA were interchangeable in both NTG and HTG sera. This implies that serum triglycerides ≤20 mmol/L (1770 mg/dL) do not adversely affect apo test results produced by LC-MS/MS, in contrast to the findings of Langlois et al. in direct HDL and LDL cholesterol tests (10). The MS-based apoA-I and apoB-100 tests described here are metrologically traceable to internationally recognized WHO-IFCC Reference Materials and Systems, which is demanded by law in medical laboratories in Europe since the European Di-

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**Fig. 4.** Linearity of apolipoprotein quantification by LC-MS/MS is illustrated for calibration samples diluted with 4% HSA in saline (●, straight line) and patient samples diluted with human serum (●, dashed line). Only the samples with the highest concentration are presented. When dilution of the sample with the highest concentration is not linear, the polynomial regression line is also presented (△).
ductive EC98/79/EC became effective in 2003. For the other apolipoproteins, internationally recognized reference materials and reference methods are not available, and value assignment of the human matrix—based, end-user’s calibrators has been performed with commercial calibrators (Fig. 1). The development of reference materials (covering the healthy and pathophysiological range) and establishment of reference methods are essential for standardization of other clinically relevant serum apolipoproteins (CM Cobbaert, personal communication, Euromedlab, Paris, June 2015).

Many of the samples selected for analysis in this study had apo concentrations above the calibration range of both LC-MS/MS and ITA. The high number of samples outside the calibration range reflects clinical practice in lipid clinics and likely explains the discrepancy between LC-MS/MS and ITA for quantification of apoC-II, apoC-III, and apoE, as a result of nonlinearity of LC-MS/MS, ITA, or both at the higher concentrations. Linearity of the LC-MS/MS assay was, nonetheless, demonstrated for concentrations up to 2-fold the calibration range and furthermore for triglyceride concentrations ≤17.3 mmol/L (1531 mg/dL).

For apoC-I, unfortunately, the applied clinical immunoassay cross-reacted with apoB; another immunoassay is therefore necessary for evaluating apoC-I method comparison in future studies. Nonetheless, the excellent linearity of the apoC-I assay by LC-MS/MS suggests that it accurately quantifies apoC-I. The results obtained with different peptides from the same apo emphasize the need to include confirmation peptides in MS-based proteomics assays at this stage of the laboratory test development. Confirmation peptides allow identification of peptide-specific instabilities, polymorphisms, mutations, or posttranslational modifications. For example, the quantification peptide for apoE provided lower concentrations than the confirmation peptide in 3 patients. In addition, the lower concentrations on the basis of the quantification peptide TPDPVSSALDK for apoC-I could be the result of the occurrence of des-TP apoC-I, a cleavage product of dipeptidyl-peptidase IV, resulting in the peptide DVSSALDK. Trenchevska et al. reported that the des-TP apoC-I proteoform is present in human plasma at approximately 25% of the total apoC-I concentration (37). For future clinical studies, samples with between-peptide inconsistencies should be reevaluated or reanalyzed to confirm the most representative peptide for quantification. It should furthermore be emphasized that the stability data from this study assumed stability of samples stored at ~80 °C. The stability of the apolipoproteins in serum stored at ~80 °C (including the value-assigned calibrators) should therefore be specifically evaluated before clinical validation.

Although the apoB-48 concentration in human serum is typically extremely low or nonexistent compared with the apoB-100 concentration, a slight discrepancy was observed between total apoB and apoB-100 measurements in HTG sera, as demonstrated by the difference between the slopes in Fig. 2. The amount of coquantified apoB-48 by ITA depends on the specificity of the antibodies used, which are typically generated for apoB-100. Therefore, it is not surprising that LC-MRM-MS measurement of total apoB shows a slightly positive bias compared to ITA in HTG sera, whereas LC-MRM-MS measurement specific for apoB-100 shows a better agreement over the entire concentration range. The specific quantification of non-apoE2 by the presented LC-MRM-MS assay can provide insight into the distribution of apoE2 concentrations compared with apoE3 and apoE4 in patient outcome studies, and can furthermore support the identification of patients with apoE2/2 phenotype, who have high CVD risk. Notably, the relative population frequencies observed in this study correlated to known prevalences (frequency for apoE3, 60%–70%; apoE4, 15%–20%; and apoE2, 5%–10%) (3). A limitation, however, is that the apoE phenotypes are not yet validated with an independent apoE phenotyping or genotyping procedure. Assessment of diagnostic sensitivity and specificity is planned in parallel with future clinical trials.

In conclusion, MS-based clinical proteomics is a valuable tool for improving our understanding of the molecular basis of CVD and, as such, contributes by identifying better treatment options for dyslipidemic patients. Future clinical trials should explore the added value of this panel of serum apolipoproteins and apoE phenotypes for CVD risk on patient outcome compared with conventional serum lipids and lipoproteins. A multiplexed LC-MS/MS assay enables visualization and elucidation of the metabolic interplay between various apolipoproteins and other lipids with regard to CVD risk, as exemplified in a heatmap of relative apolipoprotein distributions among the studied sample cohort (see online Supplemental Fig. 5). The multiplexed LC-MS/MS assay for serum apolipoproteins with semiautomated sample preparation demonstrates analytical performance characteristics that are interchangeable with those of uniplex clinical immunoassays with Conformité Européenne marking that are routinely used in medical laboratories. This type of targeted MS-based clinical proteomics assay provides complementary clinical information regarding the pathophysiology and personalized treatment options for dyslipidemia in CVD patients.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.
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