Simultaneous LC/MS/MS quantification of eight apolipoproteins in normal and hypercholesterolemic mouse plasma

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Abstract Apolipoproteins are major structural and functional constituents of lipoprotein particles. As modulators of lipid metabolism, adipose tissue biology, and energy homeostasis, apolipoproteins may serve as biomarkers or potential therapeutic targets for cardiometabolic diseases. Mice are the preferred model to study metabolic disease and CVD, but a comprehensive method to quantify circulating apolipoproteins in mice is lacking. We developed and validated a targeted proteomics assay to quantify eight apolipoproteins in mice via proteotypic signature peptides and corresponding stable isotope-labeled analogs. The LC/MS/MS method requires only a 3 µl sample volume to simultaneously determine mouse apoA-I, apoA-II, apoA-IV, apoB-100, total apoB, apoC-I, apoE, and apoJ concentrations. ApoB-48 concentrations can be calculated by subtracting apoB-100 from total apoB. After we established the analytic performance (sensitivity, linearity, and precision) and compared results for selected apolipoproteins against immunoassays, we applied the method to profile apolipoprotein levels in plasma and isolated HDL from normocholesterolemic C57BL/6 mice and from hypercholesterolemic Ldlr-/- mice.

In conclusion, we present a robust, quantitative LC/MS/MS method for the multiplexed analysis of eight apolipoproteins in normal and hypercholesterolemic mouse plasma. J. Lipid Res. 2019. 60: 900–908.

Supplementary key words animal models • lipoproteins • liquid chromatography • mass spectrometry • proteomics • quantitation

Alterations of plasma lipoproteins, especially elevated levels of apoB-containing lipoproteins, have been causally related to the risk of atherosclerotic CVD (ASCVD) (1). Apolipoproteins are major structural and functional components of lipoproteins with diverse biologic functions. They can serve as cofactors for enzymes and ligands for cell-surface receptors (2). Thereby, apolipoproteins are intimately involved in the metabolism of plasma cholesterol and triglycerides carried by lipoproteins. Blood concentrations of apoB and apoA-I, as surrogate measures for VLDL/LDL and HDL, have been proposed as superior markers for the assessment of cardiovascular risk in several trials (3, 4). In addition, apolipoproteins like apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE that can associate with various lipoprotein classes are also key players in lipid metabolism and may represent targets for the diagnosis and treatment of dyslipidemias and ASCVD (5–7). In this context, Pechlaner et al. (8) recently reported associations of several apolipoproteins (including apoC-II, apoC-III, apoE, apoH, and apoL1) with incident CVD in a prospective population-based study, which raises the potential prospects of apolipoprotein profiling for CVD.

Abbreviations: aa, amino acid; ApoE0, apoE-deficient mouse; ASCVD, atherosclerotic CVD; LDLR0, LDL receptor-deficient mouse; CV, coefficient of variation; IS, internal standard; LLOQ, lower limit of quantification; MRM, multiple reaction monitoring; SIL, stable isotope-labeled.

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Moreover, several apolipoproteins have been associated with metabolic functions in glucose homeostasis, insulin sensitivity, and adipose tissue biology and may thus contribute to obesity and diabetes mellitus (9–12). It is also established that apolipoproteins, such as apoJ and, in particular, apoE, contribute to the pathogenesis of Alzheimer’s disease via multiple mechanisms (13–15) and that apolipoproteins can exhibit antiinflammatory and antioxidative functions (16–18). Importantly, current knowledge on apolipoprotein physiology and their specific roles in various diseases is still incomplete, which underlines the importance to further study specific apolipoproteins in experimental settings.

The mouse is the most common experimental model to investigate metabolic disorders and CVD (19). At present, a comprehensive quantitative analysis of circulating apolipoproteins in mice models is limited by a lack of suitable assays. Common methods to measure apolipoproteins in mice are Ab-based immunoassays, which suffer from typical drawbacks, such as lack of specific Abs, cross-reactivity, and high lot-to-lot variability (20). Other limitations are the restriction to single-analyte testing and requirements of high sample volumes. On the contrary, the coupling of liquid chromatography and tandem mass spectrometry (LC/MS/MS) facilitates simultaneous quantitation of multiple proteins from low volumes of plasma with high throughput (20–22). To this end, surrogate proteotypic peptides, which are unique to the investigated proteins, are generated by enzymatic digestion, followed by LC separation and MS detection. In these targeted proteomics assays, stable isotope-labeled (SIL) peptides or proteins are used as internal standards (ISs), allowing the quantification of target proteins (23). Work from our laboratory and others has previously demonstrated that LC/MS/MS assays are suitable for multiplexed analysis of middle- to high-abundant apolipoproteins in human plasma (8, 24–26). However, a corresponding method to determine apolipoprotein levels in plasma of mice has not been described so far.

In the present study, we developed and validated a targeted proteomics assay for the simultaneous quantification of eight apolipoproteins from only 3 µl of mouse plasma. We then applied this method to study plasma apolipoprotein concentrations in WT C57BL/6 mice and two different hypercholesterolemic mouse models, the apoE-deficient (ApoE0) and the LDL receptor-deficient (LDLR0) mouse, in fasted as well as fed state.

MATERIALS AND METHODS

Chemicals and reagents

Ammonium bicarbonate, iodoacetamide, 2,2,2-trifluoroethanol, and Tris (2-carboxyethyl)phosphine were acquired from Sigma-Aldrich (St. Louis, MO). Formic acid and trifluoroacetic acid were obtained from Fluka (Buchs, Switzerland). Murine recombinant apoA1 was purchased from Sino Biological (Beijing, China). Ultra-high-performance LC/MS grade methanol, acetonitrile, and 2-propanol were obtained from Biosolve ( Valkenswaard, The Netherlands). Sequencing-grade modified trypsin was acquired from Promega (Madison, WI; catalog no. V5111). Ultrapure water from a Barnstead NANOpure water purification system (Thermo Fisher Scientific, Waltham, MA) was used.

Acquisition of murine plasma samples

Male normocholesterolemic C57BL/6 mice (JAX strain no. 000664), hyperlipidemic ApoE0 (JAX strain no. 002052), and LDLR0 (JAX strain no. 002207) mice were kept on a regular chow diet and studied at 13 weeks of age. Blood samples were collected in EDTA tubes at 9 AM (random fed) and after an overnight fast in the same mice. Blood samples were centrifuged at 3,000 g for 10 min to obtain plasma, which was then used for the assay, processed to prepare lipoprotein fractions, or directly stored at −80°C until analysis.

Lipoprotein fractions were isolated from 60 µl freshly prepared mouse plasma by sequential ultracentrifugation, as described previously (27). Subsequently, obtained lipoprotein fractions were stored at −80°C until analysis. Cholesterol and triglyceride levels were determined enzymatically using colorimetric assay kits (Roche Diagnostics). Protein concentration of isolated HDL was quantified using the Pierce BCA Kit (Thermo Fisher). All animal procedures were performed in accordance with the rules for animal care of the local government authorities and were approved by the animal care and use committee of Leipzig University as well as by the animal care committee of the Bezirksregierung Leipzig, Germany.

Peptide selection and synthesis

Proteotypic peptides were selected according to accepted selection rules for each of the eight apolipoproteins (28, 29). Peptide sequences containing cysteine or methionine residues as well as known polymorphisms and sites of posttranslational modifications were excluded. To ensure peptide specificity and to check for potential sequence overlaps, Blast searches against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed. Furthermore, proteotypic peptides were verified in MASCOT MS/MS ion searches (Matrix Science) against the SwissProt database after enhanced product ion analysis of tryptically digested plasma from LDLR0 mice. Peptides and SIL analogs thereof were synthesized by the Core Unit for Peptide Technology of the Interdisciplinary Center for Clinical Research (Faculty of Medicine, University of Leipzig). Synthesized peptides were purified to >98% using preparative reversed-phase HPLC. Purity and identity of the purified peptides were then evaluated by use of analytical HPLC and MALDI-MS. Peptide sequences are provided in supplemental Table S1. The individual peptides were stored as lyophilized aliquots at −80°C until use. At time of use, the lyophilized peptides were precisely weighed out and dissolved in 2-propanol/water (1:1, vol/vol) to prepare stock solutions at concentrations between 1 and 10 mmol/l. Peptide working standards (concentrated between 0.1 and 1 mmol/l) were then obtained by dilution of stock solutions with 100 mmol/l ammonium bicarbonate (see supplemental Table S2 for details). All peptide solutions were stored at −80°C. Storage, handling, and reconstitution of peptides followed the usage recommendations for MS-based assays (30).

Calibration

With each batch of samples, an in-house nine point peptide calibration series was carried along, which was equivalently processed as the study samples. The highest-concentrated calibrators were produced from peptide working standards. Lower-concentrated calibrators were then prepared by serial dilution thereof with 100 mmol/l ammonium bicarbonate. Calibration curves prepared in 100 mmol/l ammonium bicarbonate buffer were also tested against calibration curves prepared in plasma to establish
the parallelism of the response in buffer and in plasma matrix for the calibrators (supplemental Fig. S1). The workflow and all concentrations of the single calibrators for each apolipoprotein are summarized in supplemental Table S2. Calibration curves were plotted using analyte-to-4S peak area ratios. Linear regression was accomplished applying 1/x weighting. Data processing was performed with Multiquant 2.0 (Sciex).

Sample processing for LC/MS/MS

EDTA plasma, lipoprotein fractions, and calibrators were treated according to a previously established standardized sample-preparation protocol (24). In brief, 3 µl of study sample or calibrator were diluted 1:2 with a SIL peptide mix prepared in 100 mmol/l ammonium bicarbonate. Final IS concentrations are summarized in supplemental Table S1. Denaturation was performed using 6.9 mol/l 2,2,2-trifluoroethanol. For reduction of disulfide bonds, samples were incubated with 5 mmol/l Tris(2-carboxyethyl)phosphine for 30 min at 60°C. Subsequently, alkylation was performed for 30 min with 10 mmol/l iodoacetamide at room temperature in the dark. After 1:10 dilution with 100 mmol/l ammonium bicarbonate, 8 µg of trypsin dissolved in 50 mmol/l acetic acid was added. Tryptic digestion was performed for 16 h at 37°C. Digestion kinetics had been investigated using a plasma pool of C57BL/6j mice (n = 5), which had been incubated with trypsin for 0.5, 1, 2, 4, 8, 16, 20, and 24 h (supplemental Fig. S2). Digestion was stopped with 0.1% formic acid. Sample cleanup was performed by solid-phase extraction using 10 mg of Oasis HLB 1cc Flangless Vac Cartridges (Waters, Milford, MA) followed by spiking murine recombinant apoA-I into pooled plasma samples of C57BL/6j mice, resulting in two different concentration levels. Furthermore, the results obtained for apoE and apoB-100 from the LC/MS/MS assay were compared with commercially available immunoassays (Mabtech, 3752-iHP-2; Abcam, ab230032), which were performed according to the manufacturers’ recommendations. For method-comparison purposes, samples with intermediate apolipoprotein concentrations were prepared by mixing plasma pools of hyperlipidemic mice (LDLR0 and ApoE0) with pooled plasma of WT mice at different ratios.

Validation of assay characteristics

To assess assay precision, pooled plasma samples from C57BL/6j, LDLR0, and ApoE0 mice (n = 4–6 per strain) were used. The plasma pools were analyzed for at least five times on 1 day or for a single time on at least five consecutive working days to evaluate within- and between-day imprecision, respectively. Concentrations quantified at signal-to-noise ratios of 3 or 10 were defined as LOD or lower limit of quantification (LLOQ), respectively. To assess the influence of sample storage conditions, apolipoproteins were quantified in plasma samples stored at 4°C within 24 h or after 4 days; in plasma samples stored at −80°C for 2 years or in samples that underwent multiple freeze-thaw cycles (n = 3) over 6 weeks before sample preparation. Recovery of apoA-I was assessed by spiking murine recombinant apoA-I into pooled plasma samples of C57BL/6j mice, which were performed according to the manufacturers’ recommendations. To assess the influence of sample storage conditions, apolipoproteins were quantified in plasma samples of hyperlipidemic mice (LDLR0 and ApoE0) with pooled plasma of WT mice at different ratios.

Statistical analyses

Data are presented as mean ± SD unless otherwise noted. Results were analyzed by Student’s t-test (two-tailed) or by ANOVA with Dunnett’s posttest, when multiple comparisons were made. The influence of sample storage conditions and freeze-thaw cycles were evaluated using acceptable change limits criteria according to ISO 5725-6 (31, 32). Method comparison with immunoassays was performed by Passing-Bablok regression and Bland-Altman plot analysis. Data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA) or MedCalc software (MedCalc Software, Ostend, Belgium) (for Passing-Bablok and Bland-Altman). Statistical significance was defined as P < 0.05.

RESULTS

Fast chromatography and MS/MS detection

We developed a high-throughput LC/MS/MS assay to simultaneously quantify apoA-I, apoA-II, apoA-IV, apoB-total, apoB-100, apoC-I, apoE, and apoJ in mouse plasma via proteotypic signature peptides and corresponding SIL analogs. Monitored peptides and respective mass transitions are presented in supplemental Table S1. The sample-preparation procedure was optimized for reproducible quantitation of murine apolipoproteins from only 3 µl of sample. ISs were added at the very beginning of the sample processing to compensate potential sample losses. The complete sample-preparation workflow is summarized in Fig. 1. Digestion kinetics of the eight investigated apolipoproteins, which are summarized in supplemental Fig. S2, confirmed that all selected signature peptides were released within 16 h of tryptic digestion. Furthermore, proteotypic peptides and SIL analogs thereof were stable during trypsin incubation.

As VLDL secreted from mouse liver contains both apoB-48 (consisting of the N-terminal 48% of apoB-100) and full-length apoB-100, we established separate assays for apoB-100 and total apoB, which further allows the calculation of apoB-48 plasma levels. For this reason, we analyzed peptide INIDIPLPLGGK [amino acids (aa) 1344-1355], which is present in apoB-48 as well as in apoB-100 and therefore determines total plasma apoB. In addition, the
apoB-100-specific peptide DLDVVNIPLAR (aa 2,739–2,749) was monitored. Hence, apoB-48 concentrations were calculated by subtracting apoB-100 from total apoB concentrations.

Evaluation of assay performance and method validity

Sensitivity and linearity of the developed LC/MS/MS assay were established in plasma samples of normocholesterolemic chow-fed C57BL/6 mice. Determined LODs were between 0.03 µmol/l for apoA-IV, apoB-100, and apoJ and 0.2 µmol/l for apoA-I (Table 1). LLOQs ranged from 0.1 µmol/l for apoJ to 0.7 µmol/l for apoC-I (Table 1). The lower limits of the quantification range were defined by the assay’s LLOQ (for apoB-100) or the expected apolipoprotein concentrations in WT mice. The upper limits of the quantification range were adapted to allow the reliable analysis of increased apolipoprotein concentrations in hypercholesterolemic mice (Table 1).

The assay’s within-day and between-day imprecision was first determined by use of a plasma pool from chow-fed C57BL/6 mice. As shown in Table 2, within-day imprecision was found between 1.2% (apoB-total) and 10.9% (apoJ), whereas between-day imprecision ranged from 4.5% (apoA-II) to 11.2% (apoC-I), with the exception of apoB-100 (31.1%). Assay precision was also evaluated in plasma pools from LDLR0 and APOE0 mice, demonstrating similar coefficients of variation in hyperlipidemic samples (see supplemental Table S3). Of note, between-day imprecision for apoB-100 was only 5.3% in LDLR0 mice, which are characterized by elevated apoB-100. Hence, the high coefficient of variation (CV) for apoB-100 in C57BL/6 mice likely resulted from the very low apoB-100 plasma concentrations at the LLOQ.

We further exemplarily evaluated the recovery of the assay by spiking pool plasma samples from C57BL/6 mice with recombinant murine apoA-I at two different concentrations. As shown in Table 3, respective recovery rates of apoA-I in murine plasma were 101.3% and 101.9%.

Finally, we compared the LC/MS/MS results with commercially available immunoassays for apoE and apoB-100 using plasma samples from normolipidemic and hyperlipidemic mice, as well as samples of intermediate concentrations prepared by mixing plasma from normolipidemic and hyperlipidemic mice at different ratios. Data obtained with LC/MS/MS and immunoassays were highly correlated and showed good concordance (Fig. 2). We observed mean differences in the Bland-Altman plot of 7.7% for apoE and 9.5% for apoB-100 for the LC/MS/MS analysis compared with the immunological analysis. The respective Passing-Bablok slopes were 1.08 (0.99–1.15; 95% CI) for apoE and 1.27 (1.21–1.35; 95% CI) for apoB-100 (Fig. 2).

Sample stability

The influence of different sample-storage conditions was evaluated by comparing results in plasma samples stored at 4°C for several days, frozen samples, and samples that underwent repeated freeze-thaw cycles. All apolipoproteins were stable in plasma stored at 4°C for 4 days (supplemental Fig. S3). Apolipoproteins were also robust to three repeated freeze-thaw cycles over a period of 6 weeks compared with baseline levels (supplemental Fig. S4). Furthermore, we determined apolipoprotein concentrations in sample aliquots that had been stored at −80°C for 2 years. Concentrations of apoA-II and apoJ were significantly lower than the analyte-specific acceptable change limits, whereas all other apolipoprotein concentrations remained within the acceptable change limits as compared with baseline (supplemental Fig. S5).

Plasma apolipoprotein profiles in C57BL/6J, ApoE0, and LDLR0 mice

After successful assessment of the method’s analytical performance, we applied the assay to profile apolipoprotein

| TABLE 1. LODs, LLOQs, and quantification ranges of analyzed apolipoproteins |
|-----------------------------|----------------|--------------|----------------|
| Protein   | LOD, µmol/l | LLOQ, µmol/l | Quantification Range |
| apoA-I    | 0.07         | 0.23         | 1.3–240          |
| apoA-II   | 0.16         | 0.53         | 1.3–240          |
| apoA-IV   | 0.03         | 0.11         | 0.2–15           |
| apoB-100  | 0.03         | 0.11         | 0.1–6            |
| apoC-I    | 0.20         | 0.67         | 0.7–27           |
| apoE      | 0.08         | 0.28         | 0.7–129          |
| apoJ      | 0.03         | 0.10         | 0.1–4.8          |

| TABLE 2. Within-day and between-day imprecision of the assay |
|-----------------------------|----------------|--------------|----------------|
| Protein   | Within-Day Imprecision (n = 10) | Between-Day Imprecision (n = 8) |
|            | Mean, µmol/l | CV, %         | Mean, µmol/l | CV, %         |
| apoA-I     | 46.07        | 5.1           | 47.68        | 6.7           |
| apoA-II    | 60.72        | 7.0           | 57.17        | 4.5           |
| apoA-IV    | 3.37         | 6.1           | 3.29         | 6.4           |
| apoB-total | 2.52         | 1.2           | 2.21         | 8.0           |
| apoB-100   | 0.22         | 8.5           | 0.13         | 31.1          |
| apoC-I     | 9.15         | 8.4           | 9.09         | 11.2          |
| apoE       | 2.77         | 7.1           | 2.80         | 7.4           |
| apoJ       | 1.98         | 10.9          | 1.91         | 6.2           |
concentrations in plasma of male WT C57BL/6 mice as well as ApoE0 and LDLR0 mice, the two most commonly used hypercholesterolemic mouse models. Plasma apolipoprotein concentrations were determined both in overnight-fasted and random fed states (Fig. 3 and supplemental Table S4). Overall, we observed only modest differences in apolipoprotein concentrations when comparing mice in fasted versus fed state in each particular model. Although plasma concentrations of chylomicron-associated apolipoproteins A-IV and C-I were consistently increased in the fed state, concentrations of apoJ were decreased in the fed state (Fig. 3 and supplemental Table S4).

Several pronounced differences were detected when comparing apolipoprotein concentrations between the three mouse strains (Fig. 3 and supplemental Table S4): Plasma concentrations of apoE were 6-fold higher in hyperlipidemic LDLR0 mice than in normolipidemic C57Bl6/J mice, likely resulting from the lack of LDLR-mediated clearance of apoE-containing lipoproteins. As expected, apoE was not detectable in the plasma of ApoE0 mice, corroborating the validity of our method. Furthermore, plasma levels of total apoB were significantly elevated in hyperlipidemic ApoE0 and LDLR0 mice to a similar extent. However, differences in the concentrations of apoB-48 and apoB-100 were observed between both mouse strains. The highest apoB-48 plasma concentrations were quantified in ApoE0 mice, where it accounted for 93% of total plasma apoB. In contrast, apoB-48 accounted for only 61% of total apoB plasma concentrations in LDLR0 mice. Moreover, apoB-100 was significantly elevated in the plasma of LDLR0 mice as compared with C57BL/6 and ApoE0 mice (Fig. 3 and supplemental Table S4).

Another hallmark of hypercholesterolemic ApoE0 mice is reduced plasma HDL cholesterol levels (33). In line with this, the plasma concentration of apoA-I and apoA-II, the most abundant apolipoproteins of HDL, were significantly lower in ApoE0 mice compared with C57BL/6J and LDLR0 mice. In contrast, apoA-IV, an apolipoprotein that is also associated with HDL, was significantly elevated in plasma from ApoE0 mice. Because apoA-IV is an exchangeable apolipoprotein circulating on chylomicrons and HDL as well as lipid-free in plasma, these differences may not be specifically attributable to HDL.

**Apolipoprotein distribution in HDL**

Finally, we applied our method to investigate the apolipoprotein profile of HDL particles, as we had observed differences in plasma concentrations of HDL-associated apolipoproteins like apoA-I, apoA-II, and apoA-IV in all three mouse models. Therefore, plasma HDL was first isolated by sequential ultracentrifugation and then subjected to LC/MS/MS-based apolipoprotein profiling. In agreement with the results obtained in plasma, concentrations of apoA-I and apoA-II were significantly lower in HDL particles from ApoE0 mice (Fig. 3). Likewise, concentrations of apoJ and apoC-I were also reduced in the HDL of these mice. In contrast, we observed that apoA-IV was significantly enriched in the HDL of ApoE0 mice when compared with WT (3.2-fold, \( P < 0.0001 \)) and LDLR0 (2.1-fold, \( P < 0.0001 \)) mice (Fig. 4), suggesting that elevated plasma apoA-IV levels in ApoE0 mice are at least partially referable to increased levels in HDL.

**DISCUSSION**

The impact of apolipoproteins on lipid metabolism, cardiovascular risk assessment, and beyond has gained considerable interest in recent years, especially because novel therapeutic strategies directed against apolipoproteins associated with triglyceride-rich lipoproteins are being explored (34–37). Thus, a more detailed investigation in animal models is essential, as this allows the interrogation of pathophysiology and biochemistry in experimental disease settings. In the present study, we developed and validated an LC/MS/MS assay capable of measuring eight apolipoproteins simultaneously from just 3 µl of mouse plasma. The presented method was suitable to quantify apolipoproteins in plasma and isolated lipoproteins (HDL) of WT C57/BL6 mice and hypercholesterolemic LDLR0 and ApoE0 mice. This may be of particular interest, as profiling of apolipoprotein concentrations within lipoproteins may allow a more detailed view on pathophysiology associated with specific apolipoproteins.

Although MS has been applied to characterize the proteome of murine lipoprotein particles (38–40), up to now, the quantitative analysis of circulating apolipoproteins in mice has been largely restricted to Ab-based immunoassays and their inherent limitations. These limitations include the lack of specific Abs, high variability from production lot to lot, and the requirement of larger plasma volumes, as immunoassays are mainly confined to single-analyte testing (20). Major advantages of the LC/MS/MS assay presented in this study are its independence from Abs, the ability to multiplex analytes, the requirement of very small sample volumes (3 µl), and the possibility to differentiate between apoB-48 and apoB-100. The LC/MS/MS method is also suitable for high-throughput analysis: A batch of 40 samples can be analyzed within 24 h, requiring approximately 4.5 h of hands-on time for the sample-preparation procedure and less than 8 min for the LC/MS/MS detection of the eight apolipoproteins. Plasma samples can be

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**TABLE 3. Recovery of recombinant apoA-I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Initial Conc.</th>
<th>Added Conc.</th>
<th>Expected Conc.</th>
<th>Measured Conc.</th>
<th>Recovery Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoA-I</td>
<td>48</td>
<td>20</td>
<td>68</td>
<td>69.3 (± 6.6)</td>
<td>101.9</td>
</tr>
<tr>
<td>apoA-I</td>
<td>48</td>
<td>10</td>
<td>58</td>
<td>58.8 (± 1.9)</td>
<td>101.3</td>
</tr>
</tbody>
</table>

Conc., concentration.
maintained refrigerated up to 4 days or frozen for at least 6 weeks without adverse effects on apolipoprotein quantification. However, it should be noted that we observed significantly lower concentrations of apoA-II and apoJ in plasma samples that had been frozen at −80°C for 2 years. Therefore, absolute concentrations of these apolipoproteins in plasma samples frozen over an extended period of time should be interpreted with caution, as sample integrity may be compromised. In the present study, only a single proteotypic peptide per apolipoprotein was used for quantification, which may be a potential limitation of the assay.

Despite applying stringent selection criteria and rigorous evaluation of suitable proteotypic peptides, we cannot completely rule out that presently unknown posttranslational modifications or genetic mutations leading to aa changes may interfere with quantification. However, an identical approach was previously used by our group to develop an LC/MS/MS assay to quantify 12 apolipoproteins in humans, and this assay was successfully validated against another LC/MS/MS assay, as well as in external proficiency testing (41).

The method presented here was developed for biomedical research use only, following best-practice recommendations.

Fig. 2. Comparison of LC/MS/MS with immunoassay (ELISA) for apoB-100 (A) and apoE (B) by Bland-Altman plot (upper panel) and Passing-Bablok regression (lower panel). Parameters of the Passing-Bablok regression including the 95% CI are summarized in C.
for the establishment of targeted peptide measurements by LC/MS/MS. We thoroughly validated robustness and suitability of the assay for quantitative assessments, but did not attempt to establish its true accuracy. Therefore, the reported absolute concentrations may not be truly accurate and should be carefully interpreted. These caveats equally apply to commercial-research use-only immunoassays, where trueness of absolute quantification was not established either. Notwithstanding, our LC/MS/MS assay showed good concordance with available immunoassays for mouse apoE and apoB-100. Furthermore, analytical validation of the assay revealed excellent performance and low imprecision (between-day imprecision < 15%) for all investigated apolipoproteins, with the exception of apoB-100 in C57/BL6 mice (CV 31.1%). The high CV of apoB-100 in WT mice likely resulted from the apolipoprotein’s very low plasma concentration near the assay’s LLOQ. WT mice carry the majority of plasma cholesterol in HDL and display low plasma apoB levels (42, 43). Additionally, mice show high rates of hepatic apolipoprotein B mRNA editing, and it was suggested that ~70% of the VLDL particles produced in mouse liver contain apoB-48 rather than apoB-100 (44). In fact, when precision of the LC/MS/MS assay was evaluated in atherosclerosis-prone hypercholesterolemic LDLR0 mice, which are characterized by high apoB-100 levels, the CV for apoB-100 was only 5.3%. Hence, detection of apoB-100 can be performed with excellent precision in mouse models with elevated apoB-100 plasma levels.

Fig. 3. Plasma apolipoprotein profiles in C57BL6/J, ApoE0, and LDLR0 mice. Plasma was obtained from tail vein blood after overnight fasting or in random fed state at 9:00 AM in the same mice. All mice were fed a regular chow diet. Data are presented as mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.0001.
With respect to apoB-100, LDLR0 mice also differed from ApoE0 mice. Although plasma levels of total apoB were elevated in both hypercholesterolemic mouse strains, the relative distribution of apoB-48 and apoB-100 was significantly different. In ApoE0 mice, the increase of plasma apoB was predominantly mediated by elevated apoB-48 (93% of total apoB), whereas LDLR0 mice showed a much smaller relative increase in apoB-48 (only 61% of total apoB) and a marked increase in apoB-100. These findings reflect the differences in the underlying biology leading to hypercholesterolemia in LDLR0 and ApoE0 mice. In ApoE0 mice, the absence of apoE leads to the accumulation of chylomicron and VLDL remnants that are predominantly apoB-48 containing cholesteryl ester-rich particles (45). In contrast, the predominant lipoprotein accumulating in LDLR0 mice on chow diet is the apoB-100 containing LDL (46).

ApoE0 mice also have lower HDL-cholesterol plasma levels compared with WT and LDLR0 mice. By profiling apolipoprotein concentrations of isolated HDL, we also found quantitative differences in the apolipoprotein composition of these particles. We detected lower concentrations of apoA-I and apoA-II in HDL from ApoE0 mice, whereas apoA-IV was significantly enriched in HDL from ApoE0 mice. Although apoA-IV is a major component of chylomicrons in the postprandial state, approximately 25% are transferred to HDL during subsequent metabolism, and the rest are circulating freely in plasma (6). In line with higher HDL levels of apoA-IV, total plasma levels of apoA-IV were also significantly elevated in ApoE0 mice. At present, the functional significance of these findings remains to be elucidated. ApoA-IV mediates reverse cholesterol transport, but was also demonstrated to exhibit potential antiobesity effects via modulation of glucose homeostasis and energy metabolism (6). It is tempting to speculate whether elevated apoA-IV may thereby contribute to the lower body fat as well as better glucose tolerance and insulin sensitivity described in ApoE0 mice (47–49). More extensive and detailed studies are needed to provide further insights into the physiologic role of elevated apoA-IV in ApoE0 mice.

In conclusion, we developed and validated a targeted proteomics assay for the simultaneous quantification of eight apolipoproteins in mice. Profiling of apolipoproteins in plasma and isolated HDL from C57/BL6, LDLR0, and ApoE0 mice revealed significant strain differences and established that our methodology can be applied to study the effects of genetic manipulations or dietary interventions on quantitative changes in apolipoprotein concentrations.

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