



**HAL**  
open science

## Multiplexed peptide analysis for kinetic measurements of major human apolipoproteins by LC/MS/MS

Mikael Croyal, Fanta Fall, Véronique Ferchaud-Roucher, Maud Chetiveaux, Yassine Zair, Khadija Ouguerram, Michel Krempf, Estelle Nobecourt

► **To cite this version:**

Mikael Croyal, Fanta Fall, Véronique Ferchaud-Roucher, Maud Chetiveaux, Yassine Zair, et al.. Multiplexed peptide analysis for kinetic measurements of major human apolipoproteins by LC/MS/MS. Journal of Lipid Research, 2016, 57 (3), pp.509-515. 10.1194/jlr.D064618 . hal-02429670

**HAL Id: hal-02429670**

**<https://hal.science/hal-02429670>**

Submitted on 6 Jan 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **Multiplexed peptide analysis for kinetic measurements of major human apolipoproteins by LC/MS/MS**

*Mikaël Croyal<sup>1,2</sup>, Fanta Fall<sup>1,2</sup>, Véronique Ferchaud-Roucher<sup>1,2</sup>, Maud Chétiveaux<sup>2</sup>, Yassine Zaïr<sup>2</sup>, Khadija Ouguerram<sup>1,2</sup>, Michel Krempf<sup>1,2,3\*</sup>, and Estelle Nobécourt<sup>1,2,3</sup>.*

<sup>1</sup>INRA, UMR 1280, Physiologie des Adaptations Nutritionnelles, CHU Hôtel-Dieu, 8 quai Moncousu, BP 70721, F-44000 Nantes, France

<sup>2</sup>CRNHO, West Human Nutrition Research Center, CHU, F-44093 Nantes, France

<sup>3</sup>Department of Endocrinology, Metabolic diseases and Nutrition, G and R Laennec Hospital, F-44093 Nantes, France

**\*Corresponding author:**

Pr. Michel Krempf, phone: +33(0) 240 083 073, E-mail: michel.krempf@univ-nantes.fr

**Running title:** Multiplexed assay for kinetic measurements of apolipoproteins

## **ABSTRACT**

A multiplexed assay was developed by mass spectrometry to analyze in a single run six major human apolipoproteins (Apos) involved in lipoprotein metabolism: ApoA-I, ApoA-II, ApoB100, ApoC-II, ApoC-III, and ApoE. This method was validated *in vivo* in six subjects who received a 14 hour constant infusion of [5,5,5-<sup>2</sup>H<sub>3</sub>]-L-leucine at 10 μM/kg/h. Plasma lipoprotein fractions were isolated from collected blood samples and were digested with trypsin. Proteotypic peptides were subsequently analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Enrichment measurement data were compared to those obtained by the standard method using gas chromatography-mass spectrometry (GC/MS). The required time to obtain the LC/MS/MS data was less than that needed for GC/MS. The enrichments from both methods were correlated for ApoA-I ( $r = 0.994$ ;  $p < 0.0001$ ) and ApoB100 ( $r = 0.999$ ;  $p < 0.0001$ ), and the Bland–Altman plot confirmed the similarity of the two methods. Intra- and inter-assay variability calculated for the six Apos of interest did not exceed 10.7% and 12.5%, respectively, and kinetic parameters were similar and/or in agreement with previous reported data. Therefore, LC/MS/MS can be considered as a useful tool for human apolipoprotein kinetic studies using stable isotope.

## **KEYWORDS:**

Apolipoproteins, Lipoproteins/Metabolism, Lipoproteins/Kinetics, Mass Spectrometry, Nutrition Protein, Multiplexed Assay, Stable Isotope.

## INTRODUCTION

Lipoprotein kinetic studies using radioactive or stable isotope tracers have been performed for years in humans to gain a better understanding of the mechanisms involved in lipid metabolism disturbances and related diseases (1, 2). Endogenous labeling with an amino acid tracer of apolipoprotein (Apo), a main component of lipoproteins, is commonly used assuming the kinetics of Apo represent a good estimate of those of the entire lipoprotein metabolism (1, 3). The incorporation of a labeled amino acid into the protein during its synthesis is subsequently measured over time and analyzed with a compartmental model to obtain the kinetic data (4, 5). The reference method to measure tracer enrichments involves isolation of the Apos by gel electrophoresis followed by acid hydrolysis to obtain unlabeled and labeled amino acids. Then, they are derivatized for gas chromatography-mass spectrometry (GC/MS) analysis (3). These approaches are limited to one or a small number of relatively abundant Apos and remain a time-consuming process. Kinetic studies are therefore mainly focused on the most abundant structural Apos (ApoA-I, and ApoB100) and, less on others although they have central role in lipid metabolism (ApoC-II, ApoC-III and ApoE) (6).

The combination of proteomic tools such as enzymatic proteolysis and liquid chromatography-tandem mass spectrometry (LC/MS/MS) has appeared recently to be a powerful tool to study plasma proteins (7-11). Although promising, one analytical challenge is to use this method in a single run analysis for the determination of concentrations and tracer enrichments of a significant set of plasma proteins with large differences in molecular weights or abundances (6, 11).

We recently published an LC/MS/MS method to measure simultaneously the concentration, tracer enrichment, and average size of Apo(a) (9, 12). In the present study, we aimed to describe the development and the validation of a multiplexed LC/MS/MS method, performing in a single run the enrichment measurements and the quantification of six major human Apos (ApoA-I, ApoA-II, ApoB100, ApoC-II, ApoC-III, and ApoE) in plasma samples obtained from a stable isotope kinetic study in humans.

## MATERIALS AND METHODS

**Reagents.** UPLC/MS-grade acetonitrile, water, and 99% formic acid were purchased from Biosolve (Valkenswaard, Netherlands). Ammonium bicarbonate (AB), [5,5,5-<sup>2</sup>H<sub>3</sub>]-L-leucine, dithiothreitol (DTT), iodoacetamide (IA), sodium deoxycholate (SDC), trypsin, ammonium hydroxide (NaOH), and 37% hydrochloric acid (HCl) were obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). Synthetic labeled and unlabeled peptides were purchased from Thermo Scientific Biopolymers (Einsteinstrasse, Germany).

**Subjects and sample collection.** Six overweight male subjects (aged:  $46 \pm 16$  years old, body mass index:  $31.8 \pm 1.5$  kg/m<sup>2</sup>) with hypertriglyceridemia (plasma triglycerides:  $208 \pm 57$  mg/dL) were enrolled. After an overnight fast, each subject received a bolus of 10 μM/kg <sup>2</sup>H<sub>3</sub>-leucine which allowed a faster plasma enrichment plateau, immediately followed by a constant infusion (10 μM/kg/h) of <sup>2</sup>H<sub>3</sub>-leucine for 14 h. Blood samples were collected at 0, 0.75, 1.5, 2.5, 4, 6, 8, 10, 12, and 14 h in EDTA tubes (Venoject, Paris, France), and the plasma was separated by centrifugation at 4 °C for 30 min. Plasma lipoprotein fractions, including very low-density lipoprotein (VLDL), LDL, intermediate density lipoprotein (IDL) and HDL, were separated by sequential ultracentrifugation methods (13, 14) and stored at -80 °C until analysis. The Ethics Committee of Nantes University Hospital approved the clinical protocol, and a written informed consent was obtained from each subject (reference trial number: NCT01216956).

**Selection of peptide markers.** Apo sequences were BLAST searched using the UNIPROT tool ([www.uniprot.org](http://www.uniprot.org)), and theoretical proteotypic peptides were searched using the free software peptide mass calculator ([http://web.expasy.org/peptide\\_mass](http://web.expasy.org/peptide_mass)). The peptide candidates were selected to maximize sensitivity, specificity, and stability. Therefore, peptides carrying methionine and cysteine residues were not considered due to potential oxidation and peptides having less than 7 amino acids were excluded. Furthermore, peptide candidates have to contain at least one leucine residue for <sup>2</sup>H<sub>3</sub>-leucine enrichment measurement. Each putative candidate was then experimentally sought in the appropriate concentrated

lipoprotein fraction and then characterized by liquid chromatography-high resolution mass spectrometry (LC/HRMS).

**Sample preparation for LC/MS/MS analysis.** The plasma lipoprotein fractions (100  $\mu$ L) were desalted and concentrated with 50 mM AB buffer (pH 8; 3 mL) and a 5,000-Da molecular weight cut-off filter. The concentrated samples (100  $\mu$ L) were mixed with 50 mM AB buffer (pH 8; 88  $\mu$ L), 10% SDC (10  $\mu$ L), and 500 mM DTT (2  $\mu$ L). The samples were reduced for 30 min at 60 °C, then alkylated with 2  $\mu$ L of fresh IA solution (1 M in 1 M NaOH) for 60 min at room temperature, and protected from light. The samples were digested overnight with 10  $\mu$ L of trypsin solution (0.1 mg/mL in 1 mM HCl), and 10  $\mu$ L of 20% formic acid was added to stop the reaction and to precipitate the SDC. Finally, the samples were centrifuged at 15,000  $g$  and 4 °C for 15 min, and the supernatants (150  $\mu$ L) were transferred to vials for LC/MS/MS analyses. Apo were quantified in plasma and plasma lipoprotein fractions as previously described (12) and using synthetic proteotypic peptides for standard solutions and labeled [ $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ ]-K or [ $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ ]-R synthetic peptides as internal standards (**Table 1**). Apo quantification was achieved in 3 replicates and at 3 kinetic time points (baseline, 6h and 14h).

**LC/MS/MS parameters.** Peptides candidates were identified and characterized using a LC/HRMS system composed of a Synapt G2 HDMS<sup>®</sup> Quadrupole-Time-of-flight (Q-TOF) mass spectrometer (Waters Corporation, Milford, MA, USA) with an electrospray ionization (ESI) interface and an Acquity H-Class<sup>®</sup> UPLC<sup>™</sup> device (Waters Corporation, Milford, MA, USA). High throughput analyses were then performed on a Xevo<sup>®</sup> Triple-Quadrupole mass spectrometer with an ESI interface equipped with an Acquity H-Class<sup>®</sup> UPLC<sup>™</sup> device. Data acquisition and analyses were performed with MassLynx<sup>®</sup> and TargetLynx<sup>®</sup> software, respectively (version 4.1, Waters Corporation, Milford, MA, USA). Labeled and unlabeled peptides were separated on an Acquity<sup>®</sup> BEH C<sub>18</sub> column (2.1  $\times$  100 mm, 1.7  $\mu$ m, Waters) at 60 °C with a linear gradient of mobile phase B (acetonitrile containing 0.1% formic acid) in mobile phase A (5% acetonitrile in water containing 0.1% formic acid) and at a flow rate of 600  $\mu$ L/min. Mobile phase B

was linearly increased from 1% to 50% for 5 min, kept constant for 1 min, returned to the initial condition over 1 min, and kept constant for 1 min before the next injection. Ten microliters of each sample was injected into the LC column. Labeled and unlabeled peptides were then detected by the mass spectrometer with the ESI interface operating in the positive ion mode (capillary voltage, 4 kV; desolvation gas (N<sub>2</sub>) flow and temperature, 1000 L/h and 400 °C; source temperature, 120 °C). The MRM mode was applied for MS/MS detection, and the parameters were optimized for each peptide from synthetic peptide solutions. Selected MRM transitions, cone voltages, and collision energies are described in **Table 1**.

**Conventional GC/MS method for ApoA-I and ApoB100 kinetic measurements.** Isolation and measurement of leucine enrichment in ApoB100 and ApoA-I were described previously (14, 15). Briefly, ApoB100- and ApoA-I-containing lipoprotein fractions were isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then hydrolyzed with HCl. Amino acids were purified by cation exchange chromatography, derivatized (N-Propanol-acetylchlorid and heptafluorobutyric acid), and analyzed by GC/MS to determine [5,5,5-<sup>2</sup>H<sub>3</sub>]-leucine enrichment. ApoA-I concentrations were measured by immunonephelometry (Behring, Rueil Malmaison, France), and ApoB100 concentrations were obtained by selective precipitation and GC/MS with norleucine as internal standard (16).

**Data management.** The M3/M0 ratios were calculated using the chromatographic peak areas, where M3 corresponds to the <sup>2</sup>H<sub>3</sub>-leucine-labeled peptide, and M0 corresponds to the unlabeled peptide. For peptides containing two leucines (ApoA-I and ApoE), two M3 isotopomers could form and be detected simultaneously by the selected MRM transitions. Their proportions were assumed to be identical, as described previously (9, 17) and the analytical signal obtained during labeled ApoA-I and ApoE detection was experimentally enhanced 2-fold by the two coeluted isotopomers. In addition, the peptide isotopologues of ApoA-I and ApoE, containing two labeled leucine residues, were not detected in our analytical conditions and were considered as negligible. Therefore, the M3/M0 ratios measured in the biological samples were corrected by dividing the primary result by two, as described previously (17). The

M3/M0 ratios measured at baseline were subtracted from the following time point ratios. Chromatographic peaks having signal-to-noise ratios below the limit of quantification of 10:1 were excluded and samples were reanalyzed using higher sample volumes before concentration step (400  $\mu$ L). Apo concentrations were calculated using calibration curves plotted from standard solutions as described previously (12). The primary results were expressed in nM and were converted to mg/dL assuming 1 mole of peptide equal to 1 mole of protein (10).

**Kinetic analysis.** Kinetic analysis was achieved using the SAAM II modeling program (Epsilon Group, Charlottesville, VA, USA). HDL-ApoA-I, HDL-ApoE and VLDL-ApoE fractional catabolic rate (FCR) were estimated from the 14 hour samples with a mono compartmental model as described previously (18, 19). We applied the same model for HDL-ApoA-II, HDL-ApoC-III and VLDL-ApoC-III according to Batal *et al.* (20) and Chan *et al.* (21), but also for HDL-ApoC-II and VLDL-ApoC-II. Kinetic data of VLDL, IDL and LDL ApoB100 were calculated using a three compartmental model as previously described (22). Plasma leucine was used as precursor pool and pool sizes were considered to be constant as no significant variation was observed on Apo concentrations at different sampling times (not shown). According to this steady state model, the FCR was equal to the fractional synthetic rate (FSR). Production rates (PR) were calculated by the product of the FCR and the pool sizes of Apos in plasma lipoprotein fractions assuming a plasma volume of 4.5% of body weight. For ApoE, ApoC-II and ApoC-III, the concentrations that were not recovered (in the bottom fractions) were considered to be predominantly HDL apolipoproteins and these amounts were mathematically added to HDL for calculation of HDL pool sizes (20).

**Validation of the multiplexed LC/MS/MS method and statistical analysis.** To assess the intra- and inter-assay variability of the LC/MS/MS method, pooled plasma lipoprotein fractions were prepared by mixing 400  $\mu$ L of plasma lipoprotein fractions from the six subjects at the following kinetic time points: baseline, 45 min, 6 h, and 14 h. These points were chosen to reach baseline, low, intermediate, and high

tracer enrichment levels, respectively. The VLDL fractions were used for ApoB100 assay validation, and the HDL fractions were used for the other Apos (i.e., ApoA-I, ApoA-II, ApoC-II, ApoC-III, and ApoE). Each pool was then divided into 18 equal fractions of 100  $\mu$ L and treated as described above. Six fractions per time point were analyzed, and the analyses were repeated on three consecutive days. The intra- and inter-assay variability of the LC/MS/MS method were calculated (coefficient of variation, CV, %) with a maximum tolerance level of 15% (23). To assess the accuracy of the LC/MS/MS method, enrichment of ApoA-I in the HDL sample and ApoB100 in the VLDL, IDL and LDL samples was measured by conventional methods using GC/MS (six subjects, 10 kinetic time points per plasma lipoprotein fraction). The paired results, baseline excluded, obtained for ApoA-I (HDL, n = 54) and ApoB100 (VLDL, IDL and LDL, n = 162) with both analytical methods were analyzed using a Pearson correlation test. A Bland-Altman plot was also generated to test the similarity of both methods accurately (24). Graphics and statistical analyses were achieved with GraphPad Prism software (version 6.0, GraphPad Software Inc., La Jolla, CA, USA).

## RESULTS

**Selection of peptide markers.** The selection of specific peptide markers was performed successfully for each target Apo by LC/HRMS. The *in silico* selection of the proteotypic peptides led to 2 (ApoA-II and ApoC-III), 3 (ApoC-II), 16 (ApoE), 19 (ApoA-I), and 73 (ApoB100) peptide candidates. The most specific and detectable of them were selected to optimize the assay sensitivity and specificity. As shown in **Table 1**, each candidate was detected as a doubly charged precursor ion, except for ApoC-II. After MS/MS fragmentations, each precursor ion yielded between 10 (ApoE) and 29 (ApoC-III) specific and singly charged product ions (**Supplemental Figure S-1A, S-1B**). As an example, for ApoA-I and ApoB100, the complete characterization of the fragmentation patterns identified the peptide sequences underlying their specificity. The most intense product ion was then selected for MRM transitions leading

to the specific detection of the target peptides (**Supplemental Figure S-1C**). As illustrated for ApoE (**Supplemental Figure S-2**), the MRM mode allowed the specific detection of both labeled and unlabeled target peptides. As expected, the chromatographic peak intensities corresponding to the labeled ApoE peptide (M3) increased during the course of the labeled tracer perfusion.

**Comparison of LC/MS/MS with conventional GC/MS methods.** As illustrated in **Figure 1A**, the data obtained with the two methods were not statistically different and were significantly correlated with a slope close to one for ApoA-I in HDL ( $r = 0.994$ ,  $p < 0.0001$ ,  $y = 0.99x + 0.01$ ) and ApoB100 in VLDL/IDL/LDL ( $r = 0.999$ ,  $p < 0.0001$ ,  $y = 1.001x + 0.013$ ). For the Bland-Altman plot, the mean difference and the limits of agreement, corresponding to the 95% confidence level (i.e.,  $\text{mean} \pm 1.96 \times \text{SD}$ ), were drawn (**Figure 1B**), and 94% and 98% of the points were between the limits of similarity for ApoA-I and ApoB100, respectively.

**Validation of the LC/MS/MS method.** The accuracy of the LC/MS/MS method was established by comparing the ApoA-I and ApoB100 enrichment measurements with those obtained by the reference methods used for many years as described above. The precision of the LC/MS/MS measurements was determined by CVs calculated from six replicates per enrichment level and over three distinct experiments. The intra- and inter-assay variability did not exceed 10.7% and 12.5%, respectively, for any of the Apos or for various enrichments ranging from 0.04% to 7.72% (**Supplemental Table S-1**). Finally, the LC/MS/MS efficiency is illustrated in **Figure 2**, which shows the kinetic enrichment curves of the six Apos. The kinetic measurements were assessed simultaneously in 240 samples within one week (6 subjects, 10 kinetic time points and 4 plasma lipoprotein fractions).

**Kinetic parameters of Apos.** Apos were successfully quantified in plasma and lipoprotein fractions by LC/MS/MS. Total recoveries ( $\text{mean} \pm \text{SEM}$ ) of ApoE, ApoC-II and ApoC-III in plasma lipoprotein fractions separated by ultracentrifugation were  $69.7 \pm 2.5\%$ ,  $79.1 \pm 6.9\%$  and  $67.5 \pm 2.7\%$ , respectively,

and ranging from 96.2% to 104.7% for ApoA-I, ApoA-II and ApoB100. As CVs did not exceed 12.4% and Apo concentrations were stable throughout the kinetics, the mean values were kept as final concentrations (**Table 2**). ApoA-I/ApoA-II concentration ratio was of  $4.0 \pm 0.7$  in HDL and ApoC-III/ApoC-II concentration ratios were of  $3.3 \pm 0.3$  and  $3.3 \pm 0.9$  in HDL and VLDL, respectively. As shown in **Table 2**, ApoA-I and ApoB100 concentrations measured by LC/MS/MS and conventional methods were not different. Kinetic parameters of Apos were also similar with both methods for ApoB100 and ApoA-I. Compared to previous reported data, the results obtained for ApoA-II, ApoC-II, ApoC-III and ApoE were in similar ranges (**Table 2** and **Supplemental Table S-2**).

## DISCUSSION

The current approaches with the conventional GC/MS method to measure the Apo kinetics of lipoproteins using stable isotope-labeled tracers are often limited to a small number of relatively abundant proteins such as ApoB100 and ApoA-I and require a complex and time-consuming preparation. This study demonstrated that enzymatic proteolysis and subsequent LC/MS/MS analysis can overcome this pitfall.

The choice of the peptide candidates is a critical point to conduct apolipoprotein measurements by LC/MS/MS. They must be selected to not interfere with other non-targeted proteins. For example, the amino acid sequence of ApoB48 is 48% identical to that of the N-terminal ApoB100 sequence (25). The trypsin digestion of ApoB100 yields a set of peptides indistinguishable from those generated for ApoB48; therefore, they must be not considered (not shown). All the candidates showed in **Table 1** meet this criteria and some of them (ApoA-I, ApoA-II and ApoE) were identical to those selected by Ceglarek *et al* (11). The high level of reproducibility (CVs < 15%) reinforced their relevance for the kinetic enrichment measurements of the targeted apolipoproteins.

We confirmed that LC/MS/MS is able to quantify accurately plasma proteins as previously published (6, 8, 10, 11). We measured similar ApoB100 and ApoA-I concentrations whatever the analytical method previously employed. For the other Apos, the concentrations were in the same range compared with other studies involving either LC/MS/MS (6, 8, 11) or conventional methods (5, 14, 15,

18-22, 26-28). In addition, ApoA-I/ApoA-II and ApoC-III/ApoC-II concentration ratios were also in the reported ranges (6, 11, 19, 21, 26). However, some drawbacks may be encountered when using LC/MS/MS. The use of proteotypic peptides involves an optimal proteolysis to obtain a full recovery otherwise the concentrations could be underestimated. Using our protocol and an overnight trypsin digestion, the hydrolysis was complete for each target protein (6, 9-11). LC/MS/MS detector responses could also be altered by matrix effects. It was unfortunately impossible to accurately quantify Apos directly in desalted samples of lipoproteins (5,000-Da molecular weight cut-off filter) because we did not find solution to assess total Apo recoveries during the desalting process. For quantification, samples were therefore assayed separately and the sample dilution (1/25) minimized matrix effects (12) without affecting significantly the limit of quantification of the Apos analyzed in this study (signal-to-noise ratio > 10 in each sample). LC/MS/MS is based on proteotypic peptide analysis and results are primarily expressed as mol/L. To convert concentrations in mg/dL, protein molecular weights are used assuming a single polymorphic isoform for each Apo. This is not the case for ApoE and ApoC-III (20). The accurate detection of isoforms could be assessed by LC/MS/MS (10, 12) but this is a complex analysis. We have assumed that these polymorphic variations did not alter significantly the molecular weights of the targeted Apos. This is another limitation of the LC/MS/MS but the comparison with the other methods used for concentration measurements is reassuring and suggests that this pitfall is probably not critical. The protocol used (primed and constant infusion of the tracer) is another limitation as tracer boluses are often preferred for proteins with slow turnover rates (29). With this latter study design, the peak enrichments are different compared to the range we have analyzed. Additional studies are warranted to validate this LC/MS/MS method with the bolus tracer study design.

To compare and validate our LC/MS/MS method with the conventional GC/MS method for enrichment measurements, we used 240 biological samples (6 subjects, 10 kinetic time points, 4 plasma lipoprotein fractions). From isolation by gel electrophoresis (VLDL-, IDL-, LDL-ApoB100 and HDL-ApoA-I) to GC/MS processing, it took 3 weeks to obtain results, while the LC/MS/MS results were obtained within 5 days. We acknowledge that this observation relied on the laboratory technical resources

but the LC/MS/MS method was also able to provide measurements for additional Apos (**Figure 2**), which would have required some additional time with the conventional method. As suggested above, one limitation of the LC/MS/MS method measurements was the loss of accuracy for chromatographic peaks having signal-to-noise ratios below 10. For the 6 Apos studied, only 7 samples (5 VLDL and 2 IDL samples, ApoB100 only) needed to be reanalyzed. The problem was overcome by using higher sample volumes (400  $\mu$ L) during the concentration step. As shown by the Bland and Altman analysis, the enrichments measured by LC/MS/MS in this study were identical of those measured with the conventional GC/MS method and were in the same range of those reported previously by others using similar stable isotope protocols and the GC/MS method for ApoA-I (15, 26) and ApoB100 (20, 27). Similar results have been also reported for ApoA-II (21, 26), ApoE (19, 20), and ApoC-III (20, 28). To the best of our knowledge, no data have been published for ApoC-II with GC/MS measurements.

Kinetics of Apos were in good agreement with those previously published in several studies (see **Supplemental Table S-2**. For HDL-ApoA-I and ApoB100 containing lipoproteins, FCR and PR were similar whatever the analytical method employed and in the same range than those already published (5, 14, 18-22, 26, 27). In addition, HDL-ApoA-II FCR were close to those obtained for HDL-ApoA-I and ensuing PR were also consistent with previous estimates (21, 26). Similar findings were observed for ApoE and ApoC-III (19, 20, 28) although the patients studied and the study design were not perfectly comparable. As previously explained (28) and unlike ApoE (19, 20), ApoC-III FCR calculated in VLDL and HDL were similar supporting rapid exchanges of ApoC-III between both particles. We found similar data for ApoC-II but, to our knowledge, no previous work can support this finding. Despite the lack of direct comparisons in our study with the conventional GC/MS method for ApoA-II, ApoC-II, ApoC-III and ApoE, the comparison of our kinetic data with already published works supported the efficiency of the LC/MS/MS method.

Finally, we could easily add the kinetics analysis for Apo(a) that we have reported previously (12) to this multiplexed LC/MS/MS analysis. Along with others, we have shown recently that LC/MS/MS can also be used to quantify plasma proteins and assess protein sequence modifications such as polymorphic

size (6, 10-12). We have focused in this study on the concentrations and the tracer enrichments of 6 major human Apos, measured using a simple and fast protocol. These analytical methods could be merged and adapted continually to obtain, in a single run, the concentrations, polymorphic modifications, and stable isotope enrichments of most of the human Apos, making LC/MS/MS a useful tool for human lipoprotein kinetic studies.

## **ACKNOWLEDGMENTS**

We are grateful to the Biogenouest CORSAIRE core facility for their financial support. The authors thank the staff of the Clinical Investigation Center of the University Hospital in Nantes, especially Eliane Hiverneau for her invaluable help with patients and blood collection. We also thank Stéphanie Billon-Crossouard and Audrey Aguesse for their technical assistance for developing this method.

## REFERENCES

1. Foster, D. M., P. H. Barrett, G. Toffolo, W. F. Beltz, and C. Cobelli. 1993. Estimating the fractional synthetic rate of plasma apolipoproteins and lipids from stable isotope data. *J Lipid Res.* **34**: 2193-2205.
2. Lam, S. M., and G. Shui. 2013. Lipidomics as a principal tool for advancing biomedical research. *J Genet Genomics.* **4**: 375–390.
3. Lichtenstein, A. H., J. S. Cohn, D. L. Hachey, J. S. Millar, J. M. Ordovas, and E. J. Schaefer. 1990. Comparison of deuterated leucine, valine, and lysine in the measurement of human apolipoprotein A-I and B-100 kinetics. *J Lipid Res.* **31**: 1693-1701.
4. Barrett, P. H., and G. F. Watts. 2003. Kinetic studies of lipoprotein metabolism in the metabolic syndrome including effects of nutritional interventions. *Curr Opin Lipidol.* **14**: 61-68.
5. Matthan, N. R., S. M. Jalbert, S. Lamon-Fava, G. G. Dolnikowski, F. K. Welty, H. R. Barrett, E. J. Schaefer, and A. H. Lichtenstein. 2005. TRL, IDL, and LDL apolipoprotein B-100 and HDL apolipoprotein A-I kinetics as a function of age and menopausal status. *Arterioscler Thromb Vasc Biol.* **25**: 1691-1696.
6. Pan, Y., H. Zhou, A. Mahsut, R. J. Rohm, O. Berejnaia, O. Price, Y. Chen, J. Castro-Perez, M. E. Lassman, D. McLaren, J. Conway, K. K. Jensen, T. Thomas, G. Reyes-Soffer, H. N. Ginsberg, D. E. Gutstein, M. Cleary, S. F. Previs, and T. P. Roddy. 2014. Static and turnover kinetic measurement of protein biomarkers involved in triglyceride metabolism including apoB48 and apoA5 by LC/MS/MS. *J Lipid Res.* **55**: 1179-1187.
7. Lee, A.Y., N. A. Yates, M. Ichetovkin, E. Deyanova, K. Southwick, T. S. Fisher, W. Wang, J. Loderstedt, N. Walker, H. Zhou, X. Zhao, C. P. Sparrow, B. K. Hubbard, D. J. Rader, A. Sitlani, J. S. Millar, and R. C. Hendrickson. 2012. Measurement of fractional synthetic rates of multiple protein analytes by triple quadrupole mass spectrometry. *Clin Chem.* **58**: 619-627
8. Lassman, M. E., T. M. McLaughlin, E. P. Somers, A. C. Stefanni, Z. Chen, B. A. Murphy, K. K. Bierilo, A. M. Flattery, K. K. Wong, J. M. Castro-Perez, B. K. Hubbard, and T. P. Roddy. 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.
9. Zhou, H., J. Castro-Perez, M. E. Lassman, T. Thomas, W. Li, T. McLaughlin, X. Dan, P. Jumes, J. A. Wagner, D. E. Gutstein, B. K. Hubbard, D. J. Rader, J. S. Millar, H. N. Ginsberg, G. Reyes-Soffer, M. Cleary, S. F. Previs, and T. P. Roddy. 2013. Measurement of apo(a) kinetics in human subjects using a microfluidic device with tandem mass spectrometry. *Rapid Commun Mass Spectrom.* **27**: 1294-1302.
  10. Lassman, M. E., T. M. McLaughlin, H. Zhou, Y. Pan, S. M. Marcovina, O. Laterza, and T. P. Roddy. 2014. Simultaneous quantitation and size characterization of apolipoprotein(a) by ultra-performance liquid chromatography/mass spectrometry. *Rapid Commun Mass Spectrom.* **28**: 1101-1106.
  11. Ceglarek, U., J. Dittrich, S. Becker, F. Baumann, L. Kortz, and J. Thiery. 2013. Quantification of seven apolipoproteins in human plasma by proteotypic peptides using fast LC-MS/MS. *Proteomics Clin Appl.* **7**: 794-801.
  12. Croyal, M., K. Ouguerram, M. Passard, V. Ferchaud-Roucher, M. Chétiveaux, S. Billon-Crossouard, A. C. de Gouville, G. Lambert, M. Krempf, and E. Nobécourt. 2015. Effects of Extended-Release Nicotinic Acid on Apolipoprotein (a) Kinetics in Hypertriglyceridemic Patients. *Arterioscler Thromb Vasc Biol.* **35**: 2042-2047.
  13. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest.* **34**: 1345-1353.
  14. Ouguerram, K., M. Chetiveaux, Y. Zair, P. Costet, M. Abifadel, M. Varret, C. Boileau, T. Magot, and M. Krempf. 2004. Apolipoprotein B100 metabolism in autosomal-dominant hypercholesterolemia related to mutations in PCSK9. *Arterioscler Thromb Vasc Biol.* **24**: 1448-1453.

15. Chétiveaux, M., K. Ouguerram, Y. Zair, P. Maugère, I. Falconi, H. Nazih, and M. Krempf. 2004. New model for kinetic studies of HDL metabolism in humans. *Eur J Clin Invest.* **34**: 262-267.
16. Beghin, L., N. Duhal, P. Poulain, P. Hauw, B. Lacroix, J. M. Lecerf, J. P. Bonte, J. C. Fruchart, and G. Luc. 2000. Measurement of apolipoprotein B concentration in plasma lipoproteins by combining selective precipitation and mass spectrometry. *J Lipid Res.* **41**: 1172-1176.
17. Brunengraber, H., J. K. Kelleher, and C. Des Rosiers. 1997. Applications of mass isotopomer analysis to nutrition research. *Annu Rev Nutr.* **17**: 559-596.
18. Frénais, R., K. Ouguerram, C. Maugeais, P. Mahot, P. Maugère, M. Krempf, and T. Magot. 1997. High density lipoprotein apolipoprotein AI kinetics in NIDDM: a stable isotope study. *Diabetologia.* **40**: 578-583.
19. Bach-Ngohou, K., K. Ouguerram, R. Frénais, P. Maugère, B. Ripolles-Piquer, Y. Zair, M. Krempf, and J. M. Bard. 2005. Influence of atorvastatin on apolipoprotein E and AI kinetics in patients with type 2 diabetes. *J Pharmacol Exp Ther.* **315**: 363-369.
20. Batal, R., M. Tremblay, P. H. Barrett, H. Jacques, A. Fredenrich, O. Mamer, J. Davignon, and J. S. Cohn. 2000. Plasma kinetics of apoC-III and apoE in normolipidemic and hypertriglyceridemic subjects. *J Lipid Res.* **41**: 706-718.
21. Chan, D. C., G. F. Watts, M. N. Nguyen, and P. H. R. Barrett. 2006. Factorial study of the effect of n-3 fatty acid supplementation and atorvastatin on the kinetics of HDL apolipoproteins A-I and A-II in men with abdominal obesity<sup>1-3</sup>. *Am J Clin Nutr.* **84**: 37-43.
22. Maugeais, C., K. Ouguerram, M. Krempf, P. Maugeais, J. Gardette, E. Bigot, and T. Magot. 1996. A minimal model using stable isotopes to study the metabolism of apolipoprotein B-containing lipoproteins in humans. *Diabetes Metab.* **22**: 57-63.
23. Viswanathan, C. T., S. Bansal, B. Booth, A. J. DeStefano, M. J. Rose, J. Sailstad, V. P. Shah, J. P. Skelly, P. G. Swann, and R. Weiner. 2007. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *Pharm Res.* **24**: 1962-1973.

24. Bland, M., and D. G. Altman. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*. **327**: 307-310.
25. Jackson, K. G., and C. M. Williams. 2004. Apolipoprotein B-48: comparison of fasting concentrations measured in normolipidaemic individuals using SDS-PAGE, immunoblotting and ELISA. *Atherosclerosis*. **176**: 207-217.
26. Okubo, K., K. Ikewaki, S. Sakai, N. Tada, Y. Kawaguchi, and S. Mochizuki. 2004. Abnormal HDL apolipoprotein A-I and A-II kinetics in hemodialysis patients: a stable isotope study. *J Am Soc Nephrol*. **15**: 1008-1015.
27. Frischmann, M. E., K. Ikewaki, E. Trenkwalder, C. Lamina, B. Dieplinger, M. Soufi, H. Schweer, J. R. Schaefer, P. König, F. Kronenberg, and H. Dieplinger. 2012. In vivo stable-isotope kinetic study suggests intracellular assembly of lipoprotein(a). *Atherosclerosis*. **225**: 322-327.
28. Ooi, E. M., D. T. Chan, G. F. Watts, D. C. Chan, T. W. Ng, G. K. Dogra, A. B. Irish, and P. H. Barrett. 2011. Plasma apolipoprotein C-III metabolism in patients with chronic kidney disease. *J Lipid Res*. **52**: 794-800.
29. Barrett, P. H. R., D. C. Chan, and G. F. Watts. 2006. Design and analysis of lipoprotein tracer kinetics studies in humans. *J. Lipid Res*. **47**: 1607–1619.

## FIGURE LEGENDS

**Figure 1.** Comparison of the conventional GC/MS method and the LC/MS/MS method for  $^2\text{H}_3$ -leucine incorporation measurements in ApoA-I (HDL, n = 54) and ApoB100 (VLDL, IDL and LDL, n = 162). **(A)** Linear correlation obtained between the two methods and **(B)** comparison of the two methods using the Bland-Altman plot. For the Bland-Altman plot the average enrichment levels (%) obtained by both methods were calculated and then plotted against the difference of the two measurements

**Figure 2.** Mean changes in  $^2\text{H}_3$ -leucine incorporation over the course of the tracer infusion in six hypertriglyceridemic patients. Basal enrichment was subtracted ( $\delta$  M3/M0). Results were obtained for ApoA-I (HDL), ApoA-II (HDL), ApoB100 (VLDL, IDL and LDL), ApoC-II (VLDL, HDL), ApoC-III (VLDL, HDL), and ApoE (VLDL, HDL). Values are presented as the mean  $\pm$  standard error of the mean. M3,  $^2\text{H}_3$ -leucine labeled peptide; M0, unlabeled peptide.

**Table 1.** Summary of the analytical parameters selected for the detection of apolipoproteins

<b>Apolipoprotein</b>		<b>Peptide sequence</b>	<b>Fragment</b>	<b>Cone/collision (V)</b>	<b>MRM transition (<i>m/z</i>)</b>	<b>RT (min)</b>
ApoA-I	M0	ATEHLSTLSEK	$y_6^+$	30/25 V	608.3 → 664.3	1.6 ± 0.1
	M3	ATEHL <u>STL</u> SEK			609.8 → 664.3 + 667.3	
	IS	ATEHLSTLSE <b>K</b>			612.3 → 672.3	
ApoA-II	M0	SPELQAEAK	$y_6^+$	30/30 V	486.8 → 659.3	1.4 ± 0.1
	M3	SPE <u>L</u> QAEAK			488.3 → 662.3	
	IS	SPELQAE <b>A</b> K			490.3 → 667.3	
ApoB100	M0	NLQNNAEWVYQGAI <b>R</b>	$y_6^+$	50/30 V	888.5 → 707.4	2.7 ± 0.1
	M3	N <u>L</u> QNNAEWVYQGAI <b>R</b>			890.0 → 707.4	
	IS	NLQNNAEWVYQGA <b>I</b> R			893.5 → 717.4	
ApoC-II	M0	TAAQNLYEK	$y_4^+$	80/30 V	1037.9 → 552.3	1.4 ± 0.1
	M3	TAAQN <u>L</u> YEK			1040.9 → 555.3	
	IS	TAAQNLYE <b>K</b>			1045.9 → 560.3	
ApoC-III	M0	DALSSVQESQVAQ <b>Q</b> AR	$y_8^+$	40/35 V	858.9 → 887.5	2.0 ± 0.1
	M3	DAL <u>S</u> SVQESQVAQ <b>Q</b> AR			860.4 → 887.5	
	IS	DALSSVQESQVAQ <b>Q</b> A <b>R</b>			863.4 → 897.5	
ApoE	M0	LGPLVEQGR	$y_5^+$	25/30 V	484.8 → 588.3	2.1 ± 0.1
	M3	<u>L</u> GPLVEQGR			486.3 → 588.3	
	IS	LGPLVEQ <b>G</b> R			489.3 → 598.3	

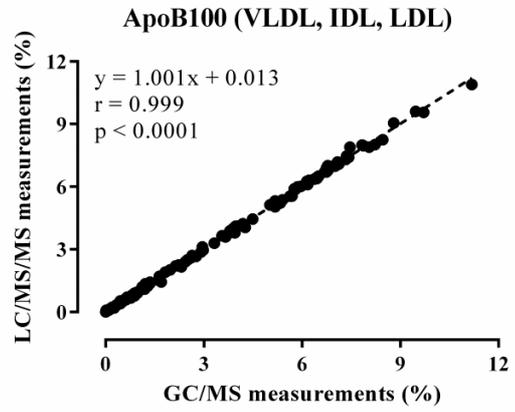
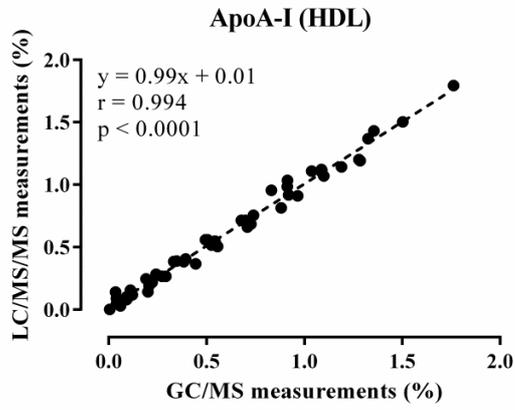
M0, unlabeled peptide; M3, labeled peptide; IS, internal standard; MRM, multiple reaction monitoring; RT, retention time. Underlined L indicates the putative incorporation site(s) of  $^2\text{H}_3$ -leucine. Bold indicates the labeled amino acid [ $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ ]-K or [ $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ ]-R.

**Table 2.** Kinetic parameters of Apos. Values are mean  $\pm$  SEM.

<b>Apolipoproteins</b>	<b>Concentration (mg/dL)</b>	<b>FCR (pool/day)</b>	<b>PR (mg/kg/day)</b>
HDL-ApoA-I, LC/MS/MS	139.0 $\pm$ 10.5	0.28 $\pm$ 0.04	17.7 $\pm$ 3.0
HDL-ApoA-I, GC/MS	136.3 $\pm$ 7.6	0.28 $\pm$ 0.04	17.2 $\pm$ 2.6
HDL-ApoA-II, LC/MS/MS	38.8 $\pm$ 6.4	0.36 $\pm$ 0.06	6.0 $\pm$ 1.0
VLDL-ApoB100 , LC/MS/MS	8.8 $\pm$ 1.1	5.44 $\pm$ 1.1	20.7 $\pm$ 2.8
VLDL-ApoB100, GC/MS	8.7 $\pm$ 1.2	5.65 $\pm$ 0.9	21.3 $\pm$ 2.8
IDL-ApoB100, LC/MS/MS	3.9 $\pm$ 0.5	5.66 $\pm$ 1.15	10.0 $\pm$ 2.5
IDL-ApoB100, GC/MS	3.7 $\pm$ 0.4	5.60 $\pm$ 1.12	9.9 $\pm$ 2.6
LDL-ApoB100, LC/MS	52.5 $\pm$ 8.3	0.30 $\pm$ 0.04	7.0 $\pm$ 1.3
LDL-ApoB100, GC/MS	49.5 $\pm$ 6.5	0.31 $\pm$ 0.05	6.8 $\pm$ 1.3
HDL-ApoC-II, LC/MS/MS	1.5 $\pm$ 0.3	1.39 $\pm$ 0.30	0.9 $\pm$ 0.2
VLDL-ApoC-II, LC/MS/MS	5.4 $\pm$ 1.5	1.44 $\pm$ 0.45	3.3 $\pm$ 1.1
HDL-ApoC-III, LC/MS/MS	4.5 $\pm$ 0.9	1.11 $\pm$ 0.12	3.2 $\pm$ 0.8
VLDL-ApoC-III, LC/MS/MS	13.2 $\pm$ 1.7	1.17 $\pm$ 0.18	13.0 $\pm$ 2.0
HDL-ApoE, LC/MS/MS	2.5 $\pm$ 0.5	0.58 $\pm$ 0.09	0.7 $\pm$ 0.3
VLDL-ApoE, LC/MS/MS	1.6 $\pm$ 0.3	2.70 $\pm$ 0.46	2.0 $\pm$ 0.6

Figure 1.

(A)



(B)

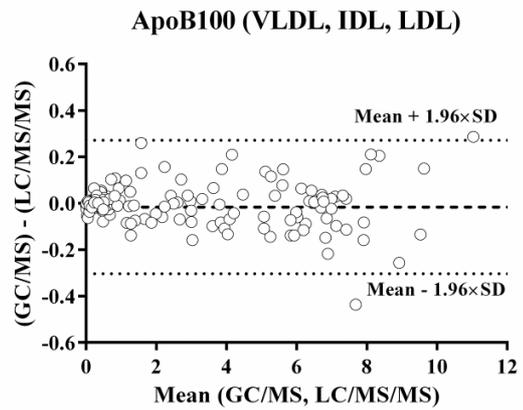
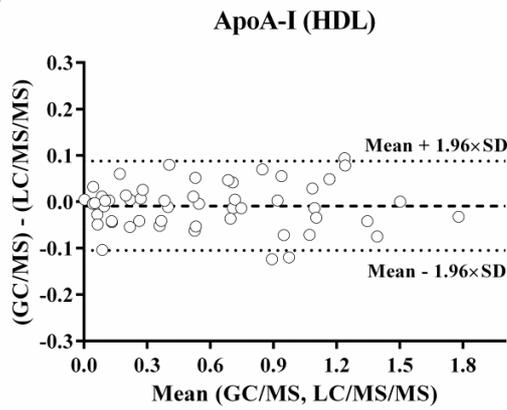


Figure 2.

