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Kinetics of plasma Apolipoprotein E isoforms by LC-MS/MS: a pilot study

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Running title: Kinetics of plasma apoE isoforms

Abbreviations: Apo, Apolipoprotein; ApoE, Apolipoprotein E; CVD, cardiovascular disease; ESI, Electrospray Ionization; FCR, Fractional Catabolic Rate; FPLC, Fast Protein Liquid Chromatography; HSPG, Heparan Sulfate Proteoglycans; LC-MS/MS, Liquid Chromatography-tandem Mass Spectrometry; LDL, Low-Density Lipoprotein; LDLR, Low-Density Lipoprotein Receptor; LRP, LDL receptor related protein; LpA, apoA-I-containing lipoproteins; LpB, apoB100-containing lipoproteins; PCSK9, Proprotein Convertase Subtilisin Kexin Type 9; PR, Production Rate; TRL, Triglyceride Rich Lipoprotein.

Abstract

Human apolipoprotein E (apoE) exhibits three major isoforms (apoE2, apoE3, and apoE4) corresponding to polymorphism in the *APOE* gene. Total plasma apoE concentrations are closely related to these isoforms but the underlying mechanisms are unknown. We aimed to describe the kinetics of apoE individual isoforms to explore the mechanisms for variable total apoE plasma concentrations. We used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to discriminate between isoforms by identifying specific peptide sequences in subjects (3 E2/E3, 3 E3/E3 and 3 E3/E4 phenotypes) who received a primed constant infusion of $^2\text{H}_3$ -leucine for 14 hours. ApoE concentrations and leucine enrichments were measured hourly in plasma. Concentrations of apoE2 were higher than apoE3, and concentrations of apoE4 were lower than apoE3. There was no difference between apoE3 and apoE4 catabolic rates and between apoE2 and apoE3 production rates, but apoE2 catabolic rates and apoE4 production rates were lower. Then, the mechanisms leading to the difference in total plasma apoE concentrations are related to contrasted kinetics of the isoforms. Production or catabolic rates are differently affected according to the specific isoforms. From these grounds, studies on the regulation of the involved biochemical pathways and the impact of pathological environments are now warranted.

Keywords: apolipoprotein E isoforms, peptide, liquid chromatography, tandem mass spectrometry, stable isotope tracers, lipoprotein/kinetics, lipoprotein/metabolism.

Introduction

Apolipoprotein E (ApoE) plays a key role in lipoprotein metabolism and, especially triglyceride-rich lipoproteins (TRL), as a ligand for the low-density lipoprotein receptor (LDLR), the LDL receptor related protein (LRP), and heparan sulfate proteoglycans (HSPG) (1-3). Human apoE is a 299 amino acid protein mostly expressed by the liver and the brain. The *APOE* gene is localized on chromosome 19 and exhibits three common alleles ($\epsilon 2$, $\epsilon 3$ and $\epsilon 4$) coding for three isoforms (apoE2, apoE3 and apoE4) that differ by single cysteine(C)-arginine(R) substitutions. ApoE3 (C₁₁₂, R₁₅₈) is the most common isoform in 50-90% of the population. ApoE2 (C₁₁₂, C₁₅₈) and apoE4 (R₁₁₂, R₁₅₈) are less frequent and found in 1-15% and 5-35% of the population, respectively (1, 2). Six phenotypes are found in humans: three homozygotes (E4/E4, E3/E3, E2/E2) and three heterozygotes (E3/E4, E2/E3, E2/E4) (2).

The LDLR, LRP and HSPG binding functions of apoE2 are reduced compared with apoE3. This may lead in the homozygote E2/E2 state to type III combined hyperlipoproteinemia and increased cardiovascular disease (CVD) risk. In contrast, apoE4 and apoE3 show similar affinities for those receptors. ApoE4 has been associated with an increased CVD risk and also appears to be a strong genetic determinant for Alzheimer disease (1, 3-5). ApoE plasma concentrations are closely related to *APOE* genotypes. Carriers of at least one $\epsilon 2$ or one $\epsilon 4$ allele respectively present with higher and lower plasma apoE levels than $\epsilon 3/\epsilon 3$ homozygotes (6-8). The mechanisms underlying these differences are still unknown.

Lipoproteins turnover can be assessed *in vivo* by measuring the incorporation of an injected tracer, usually ²H₃-leucine, in apolipoproteins over time, allowing the determination of lipoprotein kinetic parameters such as their production rates (PR) and fractional catabolic rates (FCR) (9). This approach has been improved by new analytical techniques involving enzymatic proteolysis and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (10, 11). LC-MS/MS is a powerful tool to simultaneously quantify several plasma proteins even at low concentrations (12, 13). This technique also allows the determination of protein polymorphisms (2, 14).

An LC-MS/MS method was recently developed to quantify apoE isoforms in both human plasma and cerebrospinal fluid (2). In this study, plasma apoE2 was more abundant than apoE3, and apoE3 more abundant than apoE4 in patients with $\epsilon 2/\epsilon 3$ and $\epsilon 3/\epsilon 4$ genotypes, respectively (7). To investigate why apoE isoforms concentrations differ *in vivo*, we measured the isotopic enrichment of apoE isoforms in whole plasma and in the lipoproteins to determine their kinetics in a series of $\epsilon 2/\epsilon 3$, $\epsilon 3/\epsilon 3$, and $\epsilon 3/\epsilon 4$ patients who received a primed constant infusion of $^2\text{H}_3$ -leucine.

Materials and Methods

Reagents and apparatus – UPLC/MS-grade acetonitrile, water, methanol, and 99% formic acid were purchased from Biosolve (Valkenswaard, Netherlands). $^2\text{H}_3$ -leucine was obtained from Cambridge Isotope Laboratories Inc (Andover, MA, USA). Ammonium bicarbonate was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). Synthetic labeled and unlabeled peptides were purchased from Thermo Scientific Biopolymers (Darmstadt, Germany). Stock solutions of synthetic peptides were prepared at 1 mmol/L in 50% acetonitrile containing 0.1% formic acid and stored at $-20\text{ }^\circ\text{C}$ until use. LC-MS/MS analyses were performed on a Xevo[®] TQD mass spectrometer with an electrospray (ESI) interface and an Acquity H-Class[®] UPLC[™] device (Waters Corporation, Milford, MA, USA). Data acquisition and analyses were performed with MassLynx[®] and TargetLynx[®] software, respectively (version 4.1, Waters Corporation).

Subjects and infusion protocol – Nine overweight male subjects ($3 \times \epsilon 2/\epsilon 3$, $3 \times \epsilon 3/\epsilon 3$ and $3 \times \epsilon 3/\epsilon 4$; 49 ± 11 years old; body mass index of $29 \pm 3\text{ kg/m}^2$) with hypertriglyceridemia (plasma triglycerides: $248 \pm 70\text{ mg/dL}$) were included in this study. They did not receive any treatment. After an overnight fast, each subject received an intravenous bolus of $10\text{ }\mu\text{mole/kg}$ $^2\text{H}_3$ -leucine immediately followed by a constant intravenous infusion at $10\text{ }\mu\text{mole/kg/h}$ for 14 h. Blood samples were collected hourly in EDTA tubes (Venoject, Paris, France), and the plasma was separated by centrifugation at $4\text{ }^\circ\text{C}$ for 30 min and stored at $-80\text{ }^\circ\text{C}$ until use. The Ethics Committee of Nantes University Hospital approved the clinical protocols, and

a written informed consent was obtained from each subject (trial numbers: NCT01216956 and V00002CA101).

Biochemical measurements - Cholesterol and triglyceride concentrations were measured using enzymatic kits from Boehringer Mannheim GmbH and according to supplier's instructions (Mannheim, Germany). Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) concentrations were measured in plasma by enzyme-linked immunosorbent assay according to supplier's instructions (R&D Systems, Lille, France).

Isolation of lipoproteins – Plasma lipoprotein fractions including very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL), were separated by Fast Protein Liquid Chromatography (FPLC) or by sequential ultracentrifugation methods (**15**, **16**). Total cholesterol and triglyceride contents were measured in each FPLC fraction. FPLC fractions corresponding to a same lipoprotein class were pooled. Lipoprotein fractions (2 mL for FPLC, 800 μ L for ultracentrifugation) were desalted and concentrated with 3 mL of 50 mmol/L ammonium bicarbonate buffer (pH 8) using a 5-kDa molecular weight cut-off filter for apolipoprotein enrichment measurements.

Sample preparation and proteolytic digestion - Apolipoproteins (apoA-I, apoB100, apoC-II, apoC-III, and apoE) were analyzed in plasma, lipoprotein fractions, and concentrated lipoprotein fractions using a validated multiplexed assay involving trypsin proteolysis and the subsequent analysis of proteotypic peptides by LC-MS/MS (**10**). The method was updated for the quantification of apoE isoforms as described previously (**2**). A pool solution of unlabeled synthetic peptides (M0, **Table 1**) was constituted and serially diluted in water to obtain 7 standard solutions ranging 0.5-50 μ mol/L (apoA-I), 0.25-25 μ mol/L (apoB100, apoC-II, apoC-III), and 0.1-10 μ mol/L (apoE and isoforms). Plasma, lipoprotein and standard samples (60 μ L) were reduced (addition of 120 μ L ammonium bicarbonate 50 mmol/L containing 7 mg/mL of RapidGest detergent [Waters], incubated 10 min at 80 °C; then addition of

dithiothreitol, 100 mmol/L, 20 μ L, incubated 20 min at 60 °C), alkylated (addition of iodoacetamide, 200 mmol/L, 20 μ L, incubated 20 min at room temperature in the dark) and trypsin digested overnight (5 mg/mL in HCl 1 mmol/L, 30 μ L, 37 °C) using the ready-to-use solutions of the ProteinWorks™ eXpress kit (Waters Corporation), according to the manufacturer's instructions. Labeled proteotypic peptides (**Table 1**) were used as internal standards (ISs) and a mix solution of standards was added to the digestion buffer to a final concentration of 0.5 μ mol/L. After digestion, samples were cleaned using 30 mg Oasis HLB 1 cc Cartridges (Waters Corporation). Cartridges were conditioned, equilibrated, loaded, washed and eluted with methanol (1 mL), water (1 mL), samples (~250 μ L), 5% methanol (1 mL) and 80% methanol (500 μ L), respectively. Eluates were dried under a nitrogen stream, reconstituted with 100 μ L of 5% acetonitrile containing 0.1% formic acid, and 10 μ L were injected into the LC-MS/MS system.

Analytical parameters – Apolipoprotein analyses were carried out by LC-MS/MS. Proteotypic peptides were separated over 9 min on an Acquity® BEH C₁₈ column (2.1 \times 100 mm, 1.7 μ m, Waters Corporation) held at 60°C with a linear gradient of mobile phase B (100% acetonitrile) in mobile phase A (5% acetonitrile), each containing 0.1% formic acid, and at a flow rate of 600 μ 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 2 min before the next injection. Proteotypic peptides were then detected by the mass spectrometer with the ESI interface operating in the positive ion mode (capillary voltage, 3 kV; desolvation gas (N₂) flow and temperature, 900 L/h and 400 °C; source temperature, 150 °C). The multiple reaction monitoring mode was applied for MS/MS detection as detailed in **Table 1**.

ApoE genotype validation – ApoE genotypes were confirmed by LC-MS/MS in plasma samples according to the presence of different combinations of peptides (**2**) and illustrated in **Figure 1**: E2/E2 phenotype (LGADMEDVCGR, CLAVYQAGAR), E2/E3 phenotype (LGADMEDVCGR, CLAVYQAGAR, LAVYQAGAR), E2/E4 phenotype (LGADMEDVCGR, CLAVYQAGAR, LGADMEDVR, LAVYQAGAR), E3/E3 phenotype (LGADMEDVCGR, LAVYQAGAR), E3/E4

phenotype (LGADMEDVCGR, LAVYQAGAR, LGADMEDVR), and E4/E4 phenotype (LGADMEDVR, LAVYQAGAR).

Apolipoprotein quantification – Chromatographic peak area ratios between unlabeled peptides (M0) and their respective ISs constituted the detector responses. Standard solutions were used to plot calibration curves for peptide quantification. The linearity was expressed by the mean r^2 which was greater than 0.985 for all peptides (linear regression, 1/x weighting, origin excluded). Each sample was assayed three times and the coefficients of variation did not exceed 11.3% for all peptides in all samples. Apolipoprotein concentrations were expressed in $\mu\text{mol/L}$ assuming 1 mole of peptide equivalent to 1 mole of protein. Concentrations were then converted to their standard unit (mg/dL) assuming a molecular weight of 28 079, 512 858, 8 204, 8 765 and 34 237 g/mol for apoA-I, apoB100, apoC-II, apoC-III and apoE, respectively (www.uniprot.org). For the quantification of apoE isoforms, specific CLAVYQAGAR and LGADMEDVR peptides were used for apoE2 and apoE4, respectively. Unlike apoE2 and apoE4, apoE3 isoform does not display any specific peptide. ApoE3 concentration was therefore calculated by subtracting the concentrations measured for apoE2 (E2/E3 phenotype) or apoE4 (E3/E4 phenotype) from the total apoE (LGPLVEQGR) concentration. The common peptides of apoE2/E3 (LGADMEDVCGR) and of apoE3/E4 (LAVYQAGAR) were used to confirm these apoE3 concentrations with acceptance criteria set at a maximum of 10% of variation between both approaches (2). Chemical modifications that may occur within some peptides were taken into account (secondary MRM transitions shown between parentheses, **Table 1**) and integrated to determine the exact concentrations of each apoE isoforms (2).

Enrichments of apoE isoforms – $^2\text{H}_3$ -leucine enrichments were assessed in apoE isoforms in plasma and concentrated lipoprotein fractions as previously validated (10, 14). Enrichments were calculated as described previously from unlabeled (M0) and $^2\text{H}_3$ -leucine labeled (M3) peptides (10, 17). Briefly, the isotope ratio (IR), corresponding to the M3/M0 percent ratio (%), was divided by the number of leucine residues in the peptide sequence. After baseline subtraction, IR was converted to enrichment as follows: enrichment = $(\text{IR} \times 100) \div (100 + \text{IR})$. Both apoE2 and apoE4 kinetics were investigated from their

respective signature peptides (CLAVYQAGAR and LGADMEDVR, respectively). To minimize variability, two peptides located in the same areas were used for apoE3 kinetics as illustrated in **Figure 1** (LAVYQAGAR for E2/E3 phenotype, LGADMEDVCGR for E3/E4 phenotype, and the average of both LAVYQAGAR and LGADMEDVCGR for E3/E3 phenotype). Apolipoprotein enrichment measurements were performed on 3 replicates for all kinetic time points: coefficients of variation did not exceed 12.6%.

Enrichments of total apoE – Total apoE kinetics were investigated in plasma and concentrated lipoprotein fractions by the use of the common LGPLVEQGR peptide as previously described and validated (**10**). Apolipoprotein enrichment measurements were performed on 3 replicates for all kinetic time points: coefficients of variation did not exceed 7.1%.

Precursor pool – $^2\text{H}_3$ -leucine enrichments were investigated in VLDL apoB100 (**10**). Enrichment measurements were performed on 3 replicates for all kinetic time points and coefficients of variation did not exceed 5.1%.

Kinetic parameters – Kinetic analysis was achieved using the Simulation, Analysis, and Modeling II software (SAAM II, Epsilon Group, Charlottesville, VA, USA). The labeling of apoE nearly reached the asymptotic maximal enrichment (precursor pool), which suggested a relatively rapid turnover over the time course of the study (**17**). Fractional synthetic rates (FSR) were estimated using the following mono-exponential equation: $\text{protein labeling}_{\text{time}} = \text{protein labeling}_{\text{steady-state}} \times (1 - e^{-\text{FSR} \times [\text{time-delay}]})$ (**17**). The protein labeling at steady state (precursor pool) was assumed to be close to that of a surrogate protein with fast turnover (i.e. VLDL apoB100, **Supplemental Figure S1**), and the delay parameter was set adjustable (0.01 to 1.00 hour) for calculation. As expected (fasting state), apolipoprotein pool sizes were considered constant as no significant variation was observed in apoE concentrations during the time course of the kinetic study. Production rates (PR) were calculated as the product of the FSR and of the average apoE concentration by assuming a plasma volume of 4.5% of body weight. At steady-state, the fractional catabolic rate (FCR) is equal to the FSR.

Validation of kinetic parameters – Total apoE FCR was deduced from those of specific isoforms with the following equation: $FCR_{Total} = [(Q_1 \times k_1) + (Q_2 \times k_2)] \div (Q_1 + Q_2)$. Pool sizes of isoforms 1 and 2 are expressed by Q_1 and Q_2 , respectively, and FCR of isoforms 1 and 2 are expressed by k_1 and k_2 , respectively. Kinetic parameters of total apoE were also investigated by the use of the common LGPLVEQGR peptide (**10**). Kinetic parameters obtained from both approaches (i.e., sum of isoforms vs total apoE) were then compared.

ApoE distribution within lipoproteins – Concentrations of apoE isoforms were measured in concentrated samples (60 μ L) taking into account the concentration factor. Total apoE, apoA-I (HDL) and apoB100 (VLDL/IDL/LDL) contents were measured simultaneously.

Statistical analyses – Results are expressed as mean \pm standard deviation. The nonparametric Spearman correlation test was carried out with GraphPad Prism software (version 6.0, GraphPad Software Inc., La Jolla, CA, USA) and results were considered statistically significant at $p < 0.05$.

Results

ApoE genotype validation – ApoE genotypes were confirmed by LC-MS/MS in the 9 subjects according to the presence or the absence of proteotypic peptides (**Figure 2**). Although we used a limited number of patients, precluding adequate statistical analyses, the lipid/lipoprotein/apolipoprotein levels (**Table 2**) between E2/E3, E3/E3 and E3/E4 groups were similar, including parameters PCSK9, apoB100, LDL-C, TG, apoC-II, and apoC-III. In contrast, apoE plasma concentrations appeared quite different between groups: 7.2 ± 1.1 mg/dL for E2/E3, 3.8 ± 0.7 mg/dL for E3/E3, and 3.1 ± 0.4 mg/dL for E3/E4. As shown in **Figure 3**, E3/E3 patients displayed nearly twice as much apoE3 (3.8 ± 0.7 mg/dL) than E2/E3 (2.1 ± 0.8 mg/dL) and E3/E4 (2.1 ± 0.5 mg/dL) patients. In E2/E3 individuals, apoE2 plasma concentration was higher than apoE3 (5.1 ± 0.3 vs 2.1 ± 0.8 mg/dL, respectively). In E3/E4 individuals, apoE4 plasma concentrations were lower than apoE3 (1.0 ± 0.1 vs 2.1 ± 0.5 mg/dL, respectively).

Kinetics of whole plasma apoE isoforms – Whole plasma enrichment curves of apoE3 in tracer over time were similar in E2/E3, E3/E3 and E3/E4 patients (**Figure 3**). Tracer enrichments of apoE2 were nearly half than those of apoE3 measured in E2/E3 patients (**Figure 3A**). Enrichment of apoE4 in tracer was slightly less than that of apoE3 measured in E3/E4 patients (**Figure 3C**). Of note, we did not observe any marked analytical biases for enrichment measurements obtained from both LAVYQAGAR and LGADMEDVCGR peptides (**Supplemental Figure S2**). The FCR of apoE3 were similar in E2/E3 (1.27 ± 0.31 pool/d), E3/E3 (1.51 ± 0.35 pool/day) and E3/E4 (1.63 ± 0.32 pool/d) subjects (**Figure 4**). In E3/E4 patients, apoE4 FCR (1.67 ± 0.29 pool/d) was in the same range than that of apoE3 (1.63 ± 0.32 pool/d). In E2/E3 individuals, apoE2 FCR (0.51 ± 0.05 pool/d) was lower than apoE3 (1.27 ± 0.31 pool/d). PR of apoE3 was 2.40 ± 0.21 mg/kg/d in E3/E3 subjects, and about half in E2/E3 and E3/E4 individuals (1.36 ± 0.18 and 1.52 ± 0.21 mg/kg/d, respectively), which is consistent with the presence of only one $\epsilon 3$ allele in heterozygotes. In E2/E3 individuals, the PR of apoE2 was found similar to that of apoE3 (1.16 ± 0.10 vs 1.36 ± 0.18 mg/kg/d). In sharp contrast, in E3/E4 individuals, the PR of apoE4 was twice lower than that of apoE3 (0.73 ± 0.15 vs 1.52 ± 0.21 mg/kg/d).

Validation of kinetic data – Enrichment curves of total apoE in tracer over time were also investigated with the common LGPLVEQGR peptide (**Supplemental Figure S3**). In all patients, total plasma apoE FCR and PR were on average 1.48 ± 0.29 pool/d and 3.09 ± 1.35 mg/kg/d, respectively. Total apoE FCR and PR were also calculated in all patients from kinetic data obtained specifically for each isoforms and related peptides. From these data, FCR and PR were on average 1.40 ± 0.38 pool/d and 2.54 ± 0.72 mg/kg/d, respectively. We did not find any marked difference in total apoE kinetic parameters using both approaches. As shown in **Figure 5**, there was a significant correlation between FCRs measured using both approaches ($r = 0.94$, $p = 0.001$) as well as a significant correlation between PRs ($r = 0.73$, $p = 0.031$) despite the heterogeneity in apoE2 PR.

Distribution of apoE isoforms within lipoproteins – Compared to the whole plasma concentrations, FPLC and sequential ultracentrifugations gave similar and satisfactory recovery rates for structural

apolipoproteins as apoA-I for HDL (94 and 84%, respectively) and apoB100 for VLDL, IDL and LDL (79 and 88%, respectively). A poor recovery was obtained for apoE (48 and 51%, respectively) (**Supplemental Table S1**), likely because apoE sheds off surface lipoprotein particles easily. Noteworthy, separation of IDL that are rich in apoE was not optimal (**Supplemental Figure S4**) and led to a ~130 fold dilution of the original sample by FPLC. In addition, apoE2 and apoE4 peptides displayed ~10 fold lower ionization yields than the common apoE peptide, precluding accurate detection of both isoforms in FPLC fractions despite a concentration procedure. The distribution of apoE isoforms within lipoprotein classes was therefore investigated in the non-diluted fractions obtained after ultracentrifugation. As shown in **Figure 6A**, the major apoE isoform found in apoB100-containing lipoproteins from E2/E3 individuals was apoE3 ($73.2 \pm 15.6\%$), whereas the major apoE isoform in apoA-I-containing lipoproteins was apoE2 ($66.7 \pm 22.3\%$). In contrast, the major apoE isoform present in apoB100-containing lipoproteins from E3/E4 individuals was apoE4 ($59.3 \pm 8.3\%$), whereas the major apoE isoform in apoA-I-containing lipoproteins was apoE3 ($82.0 \pm 5.6\%$) (**Figure 6B**), indicating a higher affinity of apoE2 for HDL and of apoE4 for apoB100-containing lipoproteins while apoE3 distributed homogeneously between lipoprotein classes (**Figure 6C**) in those hypertriglyceridemic patients.

Enrichments of apoE within lipoproteins – We were not able to detect $^2\text{H}_3$ -leucine enrichments of apoE isoforms in lipoprotein fractions because of insufficient sensitivity. Kinetic enrichments of total apoE were therefore investigated within lipoproteins by using the common LGPLVEQGR peptide and we did not observe any pronounced difference on total apoE kinetics between groups (**Supplemental Figure S3**). In all patients, total apoE FCR were of 0.49 ± 0.08 and 2.95 ± 0.65 pool/d, production rates were of 0.47 ± 0.12 and 2.59 ± 0.91 mg/kg/d in HDL and VLDL, respectively.

Discussion

We investigated the plasma concentrations, lipoprotein distribution and kinetic parameters of apoE2, apoE3 and apoE4 in human plasma by LC-MS/MS. Total circulating apoE concentrations and kinetics

were different according to apoE isoforms with different repartitions within lipoproteins. We showed that the differences in the whole plasma apoE isoform concentrations stemmed from a reduced clearance rate of the apoE2 isoform but from a reduced production rate of the apoE4 isoform, compared to apoE3.

One limitation of the study is the small number of subjects and the lack of $\epsilon 2/\epsilon 2$, $\epsilon 2/\epsilon 4$ or $\epsilon 4/\epsilon 4$ patients. This is related to the very low frequencies of these genotypes in our medical environment. A second limitation of our study is that we used a simple mathematical approach. Because of the small number of subjects per group, we did not develop compartment models including a delay, which might have provided a better fit to the experimental data (9). This compartmental analysis will be mandatory when more subjects will be analyzed. But a specific study is required as some patients, especially with E2 isoforms, are few and difficult to recruit. Finally, we did not use calibration solutions with known enrichments for each proteotypic peptides. This is a third limitation and we cannot totally rule out any analytical bias in assessing apoE2 and apoE4 enrichments

LC-MS/MS is reliable to simultaneously quantify several proteins (12, 13, 19), but also to study their polymorphisms (2, 14) and to measure their kinetics (10, 11). This approach involves a trypsin proteolysis before analysis of signature peptides carefully selected to maximize sensitivity, specificity, and stability. Peptide candidate selection is a crucial step unfortunately limited when considering polymorphic modifications. Here we have optimized our previous protocol (10, 12, 14) to quantify and study total apoE and each isoforms in human plasma. Despite our efforts to set up optimal proteolysis conditions (2, 20), some of our peptides displayed 10-15 fold reduced sensitivities by mass spectrometry compared with the peptide selected for total apoE measurement, likely because these peptides either contain a methionine or a cysteine residue responsible for side chains reactions and poor stability (2, 10, 21, 22). This reduced sensitivity and stability did not allow the measurement of $^2\text{H}_3$ -leucine enrichments in apoE isoforms in lipoproteins fractions. We were able to get only total apoE kinetics within lipoproteins with the common and more sensitive LGPLVEQGR peptide. Although total apoE kinetic parameters in both HDL and VLDL were in agreement with previous reports (10, 23, 24), we did not observe any marked difference

between patients with heterozygous phenotypes. To assess the kinetics of apoE isoforms, the common LGPLVEQGR peptide therefore appears limited to homozygous phenotypes.

Another hurdle, unrelated to the mass spectrometry technology, is the exchange of apoE between lipoproteins and their shedding off lipoprotein surface by ultracentrifugation, a clear bias for accurate determination of apoE pool sizes (**10, 23**). In that respect, immuno-affinity separations or softer ultracentrifugation techniques could yield better recovery rates (**12, 23, 25**). The exchangeability of apolipoproteins could also be a limitation to determine apoCs kinetic enrichment curves. However, apoC-II enrichment curves in VLDL and HDL are similar, and those of apoC-III much closer than those observed for apoE. Furthermore, apoE enrichment curves in VLDL and HDL parallel those observed for VLDL-apoB100 and HDL-apoA-I (**Supplemental Figures S1 and S3**). While apoC displayed similar kinetics in VLDL and HDL, apoE enrichment curves were sharply different between VLDL and HDL, and relatively close to those of VLDL-apoB100 and HDL-apoA-I, respectively (**10, 23**), indicating that the differences observed in apoE enrichment between lipoprotein subclasses is genuine and that apoE exchange is limited.

We observed a preferential association of apoE4 with apoB100-containing lipoproteins, in agreement with previous reports (**26-28**). The presence of a positive charge in the arginine residue at position 112 of apoE4 enhances its affinity for lipids compared with apoE3 (**26, 29**), and further strengthen its association with VLDL. In addition, the absence of cysteines at position 112 and 158 reduces apoE4 ability to establish disulfide bonds with HDL-apoA-II (**26, 30**). In contrast, we observed a preferential association of apoE2 with HDL, in line with a previous study (**31**). In contrast with apoE4, the cysteine residue at position 112 on apoE3 and at position 112 and 158 in apoE2 allows the formation of apoE/apoA-II heterodimers and could explain their preferential association within HDL compared with apoE4 (**26, 30, 32, 33**). The cysteine residue at position 158 in apoE2 alters its conformation and its ability to bind to the LDLR (**29**). Whether this might reduce apoE2 ability to associate with apoB100-containing lipoproteins is not established (**26, 31**).

As anticipated, the catabolic rate of apoE2 was slower than that of apoE3 or apoE4, as previously shown with radio-isotopes (30) or with $^{13}\text{C}_6$ -leucine in a pilot study conducted in humans (one subject from each genotype E3/E3, E3/E4, E4/E4 and E2/E4) (18). As mentioned above, the presence of a cysteine instead of an arginine at position 158 reduces the affinity of apoE2 for the LDLR by ~98% (8, 29), and its affinity for the LRP or HSPG by ~50%, compared with apoE3 (31), consistent with a reduced catabolism. Another mechanism has been proposed (34). Since the turnover of VLDL is much faster than that of HDL, the preferential distribution of apoE2 within HDL could also explain why apoE2 is cleared more slowly than apoE3 or E4. In addition, the reduced apoE2 FCR could be also explained by its association with apoA-II in HDL as detailed above. It has been suggested that both apo(E2/A-II) and apo(A-II/E2/A-II) complexes could prevent LDLR binding by masking the apoE2 component (32, 33).

We also showed that reduced apoE4 concentrations were associated with a two-fold reduction in its production rate compared with apoE3 and apoE2. This is not due to different gene expression of the three isoforms (35). However, the secretion of apoE2 and apoE4 by macrophages appears significantly reduced, compared with that of apoE3 (35), indicating that post-translational mechanisms governing apoE secretion could be related to its isoforms. Another mechanism could involve the recycling of apoE within the hepatocytes. After the initial secretion a part of apoE is submitted to a reuptake and is immediately recycled to contribute to the overall production (36). Compared with apoE3, the intracellular hepatocyte recycling of apoE4 derived from VLDL appeared to be lower (37). ApoE4 from VLDL is also apparently recycled via distinct cellular pathways (38). The precise cellular mechanisms underpinning the reduced secretion rate of apoE4 clearly remains to be elucidated.

In this study, we have evaluated a novel approach to assess the kinetic parameters of plasma apoE isoforms. We showed that the variations of total apoE plasma concentrations ($\text{E2/E3} > \text{E3/E3} > \text{E3/E4}$) associated with these phenotypes can be explained by reduced catabolic rates for apoE2 and reduced production rates for apoE4. Improvements in the sensitivity of our techniques and in our modeling approach are warranted to assess the kinetics of each apoE isoform within lipoprotein subclasses.

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Table 1 - Analytical parameters used for each proteotypic peptide.

Protein	Name	Peptide	Fragment	Cone/collision (V)	MRM (<i>m/z</i>)
ApoA-I	M0	ATEHLSTLSEK	y ₁₀ ²⁺	25/15	406.2 → 573.2
	IS	ATEHLSTLSE-[¹³ C ₆ ¹⁵ N ₄]R	y ₁₀ ²⁺	25/15	408.9 → 577.2
ApoB100	M0	ATGVLYDYVVK	y ₆ ⁺	34/23	622.4 → 915.6
	M3	ATGVLYDYVVK	y ₆ ⁺	34/23	623.9 → 915.6
	IS	ATGVLYDYVN-[¹³ C ₆ ¹⁵ N ₂]K	y ₆ ⁺	34/23	626.4 → 923.6
ApoC-II	M0	TAAQNLYEK	y ₇ ⁺	35/20	519.7 → 865.7
	IS	TAAQNLYE-[¹³ C ₆ ¹⁵ N ₂]K	y ₇ ⁺	35/20	523.7 → 873.7
ApoC-III	M0	GWVTDGFSSLK	y ₈ ⁺	40/35	598.2 → 854.1
	IS	GWVTDGFSSL-[¹³ C ₆ ¹⁵ N ₂]K	y ₈ ⁺	40/35	602.2 → 862.1
ApoE	M0	LGPLVEQGR	y ₅ ⁺	25/30	484.8 → 588.3
	M3	LGPLVEQGR	y ₅ ⁺	25/30	486.3 → 588.3
	IS	LGPLVEQG-[¹³ C ₆ ¹⁵ N ₄]R	y ₅ ⁺	25/30	489.8 → 598.3
ApoE2	M0	[C]LAVYQAGAR	y ₈ ⁺	40/22	555.2 (527.7) → 835.7
	M3	[C]LAVYQAGAR	y ₈ ⁺	40/22	556.7(529.3) → 835.7
	IS	[C]LAVYQAGA-[¹³ C ₆ ¹⁵ N ₄]R	y ₈ ⁺	40/22	560.2 → 845.7
ApoE4	M0	LGAD[M]EDVR	y ₈ ⁺	35/20	503.6 (511.6) → 892.6 (908.6)
	M3	LGADMEDVR	y ₈ ⁺	35/20	505.1 (513.1) → 892.6 (908.6)
	IS	LGADMEDV-[¹³ C ₆ ¹⁵ N ₄]R	y ₈ ⁺	35/20	508.6 → 902.6
ApoE2/E3	M0	LGADMEDV[C]GR	y ₆ ⁺	35/20	612.0 → 735.6
	M3	LGADMEDV[C]GR	y ₆ ⁺	35/20	613.5 → 735.6
	IS	LGADMEDV[C]G-[¹³ C ₆ ¹⁵ N ₄]R	y ₆ ⁺	35/20	612.0 → 735.6
ApoE3/E4	M0	LAVYQAGAR	y ₇ ⁺	40/22	475.0 → 764.7
	M3	LAVYQAGAR	y ₇ ⁺	40/22	476.5 → 764.7
	IS	LAVYQAGA-[¹³ C ₆ ¹⁵ N ₄]R	y ₇ ⁺	40/22	480.0 → 774.7

M0, unlabeled peptide; M3, ²H₃-leucine labeled peptide; IS, internal standard; MRM, multiple reaction monitoring. Parentheses indicate secondary transitions that may occur. [C] indicates carbamidomethyl-cysteine (+57) and [M] indicates oxidized methionine.

Table 2 - Clinical and biochemical characteristics of patients. Values are means \pm standard deviations.

Parameters	E2/E3	E3/E3	E3/E4
N	3	3	3
Age (y)	51 \pm 5	45 \pm 15	44 \pm 9
BMI (kg/m ²)	31 \pm 3	27 \pm 3	28 \pm 4
TC (mg/dL)	193 \pm 38	209 \pm 28	231 \pm 9
TG (mg/dL)	207 \pm 43	290 \pm 80	228 \pm 47
HDL-C (mg/dL)	41 \pm 5	41 \pm 12	40 \pm 5
LDL-C (mg/dL)	114 \pm 28	116 \pm 28	150 \pm 8
ApoA-I (mg/dL)	128 \pm 27	139 \pm 28	138 \pm 14
ApoB100 (mg/dL)	105 \pm 18	119 \pm 17	116 \pm 4
ApoC-II (mg/dL)	4 \pm 1	4 \pm 2	4 \pm 2
ApoC-III (mg/dL)	19 \pm 5	27 \pm 7	22 \pm 4
ApoE (mg/dL)	7.2 \pm 1.1	3.8 \pm 0.7	3.1 \pm 0.4
PCSK9 (ng/mL)	300 \pm 136	369 \pm 170	298 \pm 65

BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisine/kexine type 9.

Figure 1 – Selection of signature peptides from apoE sequences for isoform analysis. Phenotype identification and apoE isoform concentrations were assessed using different combinations of peptides. Both apoE2 and apoE4 carry a single specific peptide (CLAVYQAGAR and LGADMEDVR, respectively) unlike apoE3. For enrichment measurements in heterozygous patients, LAVYQAGAR and LGADMEDVCGR were used for apoE3 kinetics in E2/E3 and E3/E4 phenotypes, respectively. In homozygous E3/E3 patients, enrichments of LAVYQAGAR and LGADMEDVCGR were averaged. Blue indicates cysteine-arginine interchanges between isoforms.

Isoforms	Sequences	Peptides
ApoE2	<p>KVEQAVETEPEPELRRQQTTEWQSGQRWELALGRFWDYLRWVQTLSEQVQE ELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAA QARLGADMEDVCGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKLLR DADDLQKCLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAG QPLQERAQAWGERLRARMEEMGSRTDRDLDEVKEQVAEVRAKLEEQAQQ IRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH</p>	<p>CLAVYQAGAR LGPLVEQGR</p>
ApoE3	<p>KVEQAVETEPEPELRRQQTTEWQSGQRWELALGRFWDYLRWVQTLSEQVQE ELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAA QARLGADMEDVCGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKLLR DADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAG QPLQERAQAWGERLRARMEEMGSRTDRDLDEVKEQVAEVRAKLEEQAQQI RLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH</p>	<p>LAVYQAGAR LGADMEDVCGR LGPLVEQGR</p>
ApoE4	<p>KVEQAVETEPEPELRRQQTTEWQSGQRWELALGRFWDYLRWVQTLSEQVQE ELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAA QARLGADMEDVRGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKLLR DADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAG QPLQERAQAWGERLRARMEEMGSRTDRDLDEVKEQVAEVRAKLEEQAQQI RLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH</p>	<p>LGADMEDVR LGPLVEQGR</p>

Figure 2 – Identification of apoE phenotypes by selective combination of proteotypic peptides. LC-MS/MS chromatograms obtained in plasma from representative subjects.

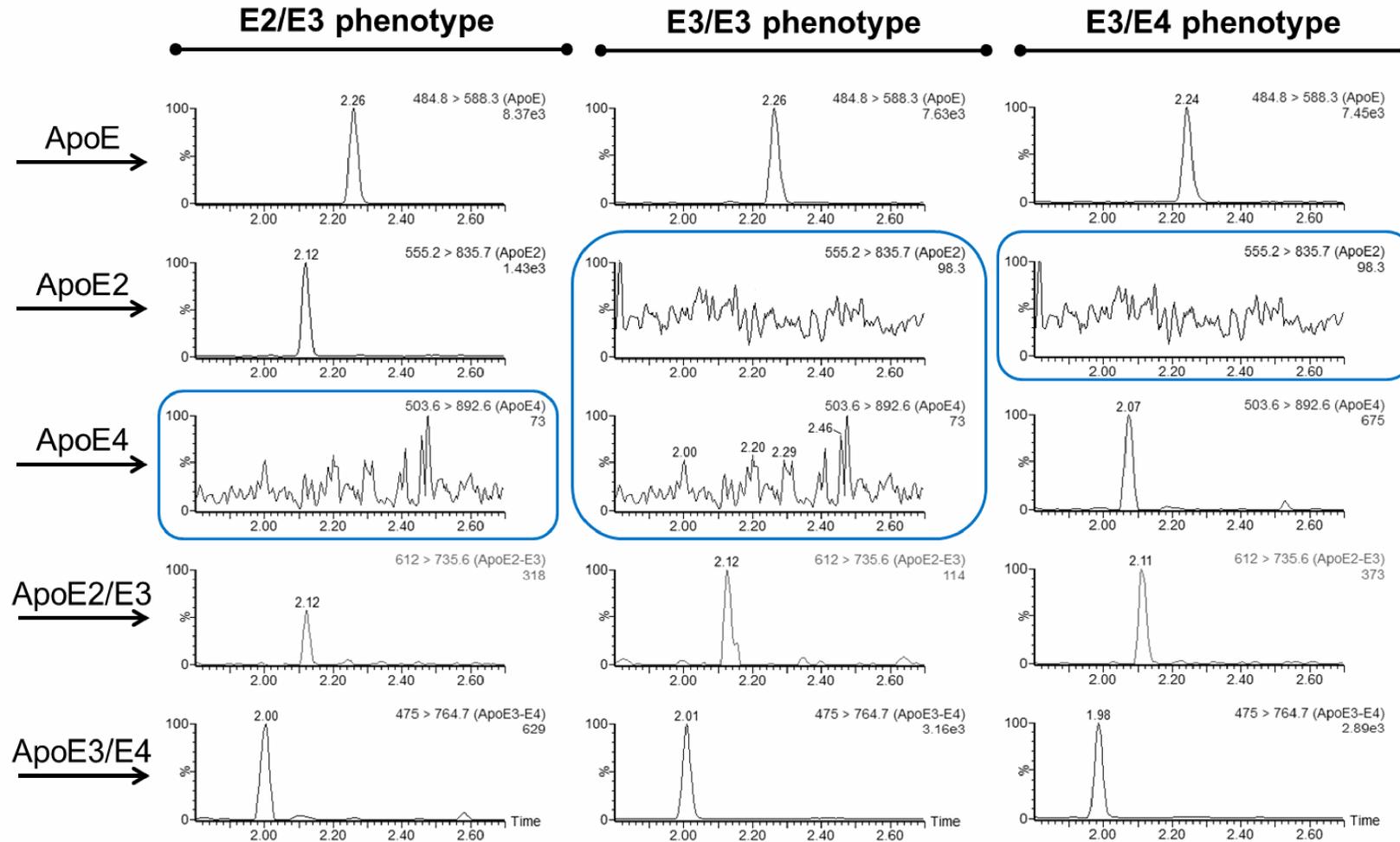


Figure 3 – Kinetics of plasma apoE isoforms. Mean plasma concentrations and mean changes in ²H₃-leucine incorporation over the course of the tracer infusion in subjects with (A) E2/E3 phenotype, (B) E3/E3 phenotype and (C) E3/E4 phenotype. Values are presented as means ± standard deviations (n = 3). Total apoE concentrations are shown as indicative in heterozygote patients.

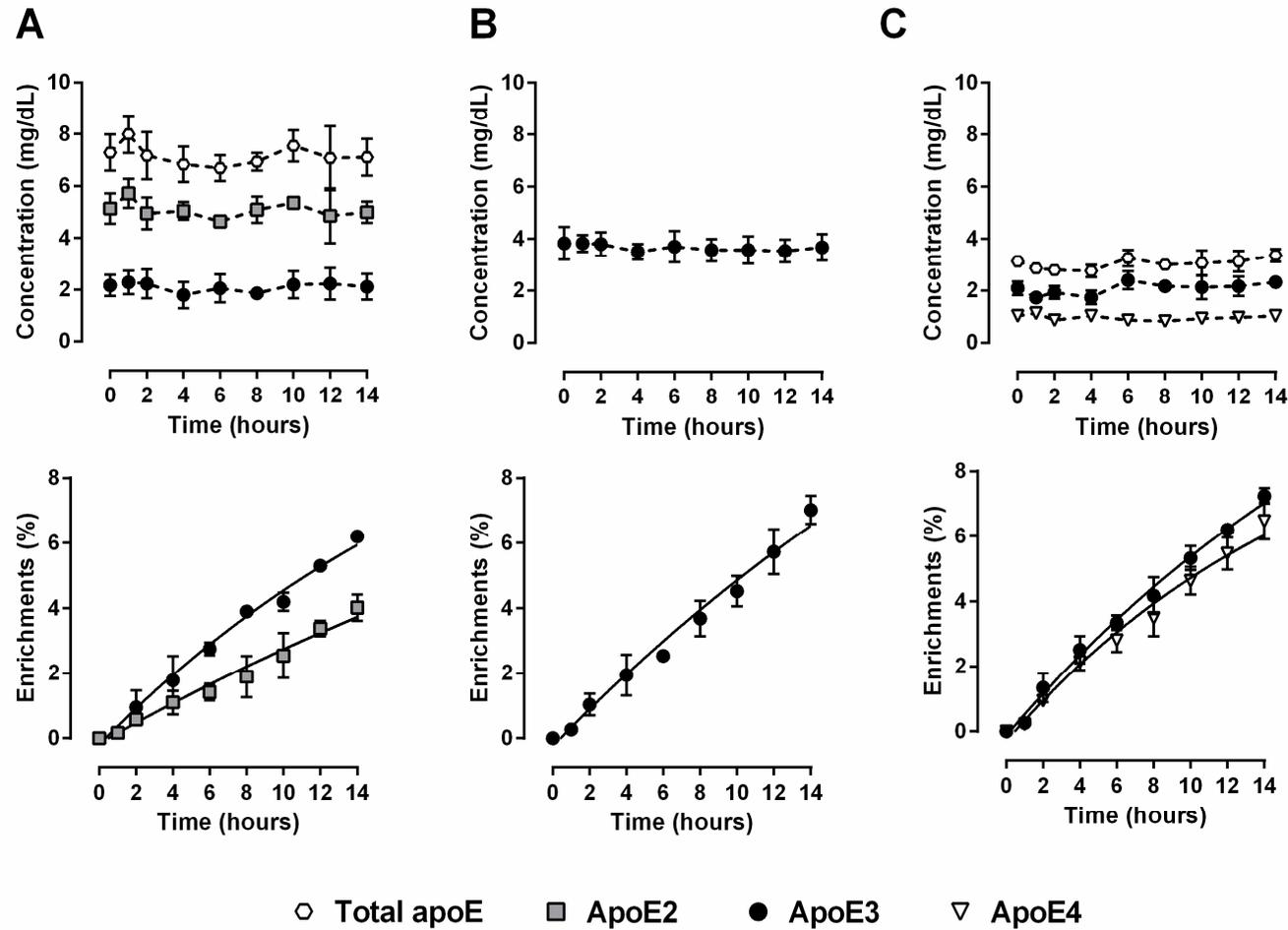


Figure 4 – Kinetic parameters of plasma apoE isoforms. (A) Fractional catabolic rates (FCR) and (B) production rates (PR) estimated with a mono-exponential equation in subjects with E2/E3 phenotype, E3/E3 phenotype and E3/E4 phenotype.

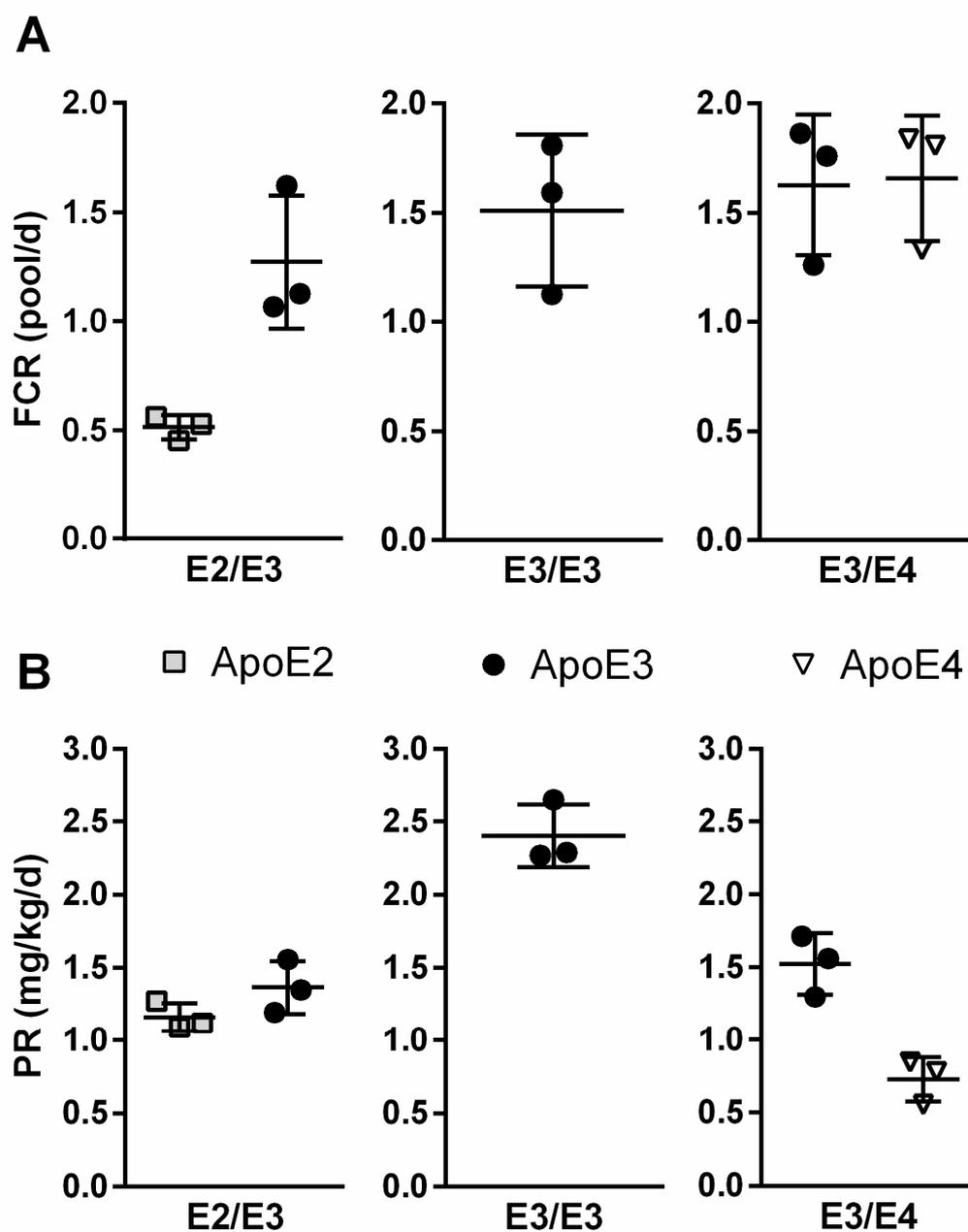


Figure 5 – Validation of plasma apoE kinetic parameters. Total fractional catabolic rates (FCR) and production rates (PR) were calculated for each subject from kinetic data of apoE isoforms, and then compared (Spearman correlation test) with those obtained directly from the LGPLVEQGR peptide used for total apoE detection. Grey, black and white circles indicate E2/E3, E3/E3 and E3/E4 phenotypes, respectively.

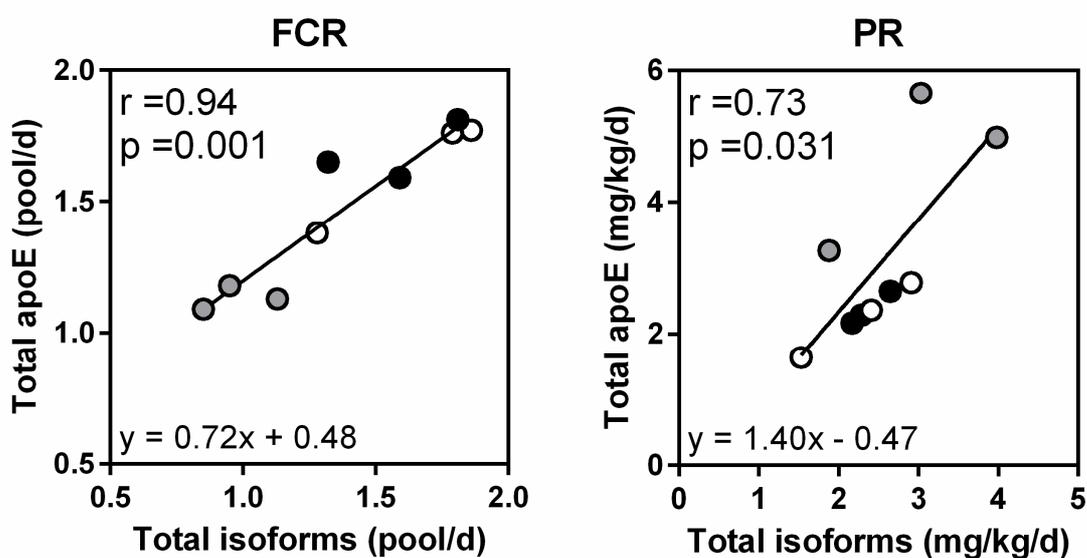


Figure 6 – Distribution of apoE isoforms within lipoproteins in hypertriglyceridemic patients. ApoE isoforms were assayed in (A) apoB100-containing lipoproteins (i.e. VLDL+IDL+LDL) and (B) in apoA-I-containing lipoproteins (i.e. HDL) obtained by ultracentrifugation. (C) Summary of apoE isoform distribution within lipoproteins. Concentrations were normalized to the total content of apoE recovered in each lipoprotein subclass. Values are means \pm standard deviations.

