Effects of Extended-Release Nicotinic Acid on Apolipoprotein (a) Kinetics in Hypertriglyceridemic Patients

Mikaël Croyal, Khadija Ouguerram, Maxime Passard,* Véronique Ferchaud-Roucher,* Maud Chétiveaux, Stéphanie Billon-Crossouard, Anne-Charlotte de Gouville, Gilles Lambert, Michel Krempf,* Estelle Nobécourt*

Objective—To determine the mechanisms by which extended-release nicotinic acid reduces circulating lipoprotein (a) concentrations in hypertriglyceridemic patients.

Approach and Results—Eight nondiabetic, obese male subjects (aged 48±12 years; body mass index, 31.2±1.8 kg/m²) with hypertriglyceridemia (triglycerides, 226±78 mg/dL) were enrolled in an 8 week, double blind, placebo-controlled cross-over study. At the end of each treatment phase, fasted subjects received a 10 µmol/L per kg bolus injection of [5,5,5-2H₃]-Leucine immediately followed by constant infusion of [5,5,5-2H₃]-Leucine (10 µmol L⁻¹ kg⁻¹ h⁻¹) for 14 hours, and blood samples were collected. A liquid chromatography–tandem mass spectrometry method was used to study apolipoprotein (a) (Apo(a)) kinetics. The fractional catabolic rate of Apo(a) was calculated with a single compartmental model using the apolipoprotein B100 (ApoB100) containing very low density lipoprotein tracer enrichment as a precursor pool. Extended-release nicotinic acid decreased plasma triglycerides (−46%; P=0.023), raised high-density lipoprotein cholesterol (+20%; P=0.008), and decreased Apo(a) plasma concentrations (−20%; P=0.008). Extended-release nicotinic acid also decreased ApoB100 (22%; P=0.008) and proprotein convertase subtilisin/kexin type 9 (PCSK9, −29%; P=0.008) plasma concentrations. Apo(a) fractional catabolic rate and production rates were decreased by 37% (0.58±0.28 versus 0.36±0.19 pool/d; P=0.008) and 50% (1.4±0.8 versus 0.7±0.4 nmol/kg per day; P=0.008), respectively.

Conclusions—Extended-release nicotinic acid treatment decreased Apo(a) plasma concentrations by 20%, production rates by 50%, and catabolism by 37%. ApoB100 and PCSK9 concentrations were also decreased by treatment, but no correlation was found with Apo(a) kinetic parameters. (Arterioscler Thromb Vasc Biol. 2015;35:2042-2047. DOI: 10.1161/ATVBAHA.115.305835.)

Key Words: apolipoprotein (a) ■ hypertriglyceridemia ■ kinetics ■ lipoprotein (a) ■ niacin

Lipoprotein (a) [Lp(a)] is a proatherogenic lipoprotein composed of a low-density lipoprotein (LDL) and apolipoprotein (a) [Apo(a)], a glycoprotein synthesized by the liver and covalently bound to the apolipoprotein B100 (ApoB100).1,2 Apo(a) is composed of a protease domain (peptidase S1) and kringle IV and kringle V domains. Kringle V, the peptidase S1 domain, and the Kringle IV (KIV) domains (KIV₁ to KIV₁₀) are expressed once, except KIV₅, which is repeated 3× to 40×. Apo(a) size is genetically determined and is highly heterogeneous among and within patients, as most individuals have 2 circulating isoforms encoded by 2 different alleles. A low copy number of KIV₅ has been associated with higher plasma Lp(a) concentration, possibly because of differences in Apo(a) production rates.1,3

Lp(a) is associated with increased cardiovascular disease.1,4 Robust epidemiological and Mendelian randomization studies support the hypothesis that increased Lp(a) plasma concentrations are related to an increased risk of coronary heart disease,5,6 even in cases of high LDL cholesterol (LDL-C).7 The European Atherosclerosis Society has proposed that the optimal Lp(a) level should be <50 mg/dL.4 Currently, there are few available options for lowering Lp(a).7-11 Lipoprotein apheresis was approved in Germany for patients with progressive cardiovascular diseases to control LDL-C levels and Lp(a). Recently, developed lipid-lowering treatments, including targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) and inhibiting cholesteryl ester transfer protein, have shown significant lowering of Lp(a).1

Extended-release nicotinic acid (ERN) is the only available oral treatment that is able to lower Lp(a) by 25% to 30%.12 In vitro studies have shown that nicotinic acid could increase retention of Apo(a) at the hepatocyte surface, and it may also...
inhibit Apo(a) gene transcription.\textsuperscript{1,13} Few human studies have looked at Lp(a) metabolism in untreated healthy volunteers,\textsuperscript{14} but, to date, there have been no human studies characterizing the effects of ERN on Lp(a) metabolism. New analytical methods based on liquid chromatography–tandem mass spectrometry and enzymatic proteolysis have simplified the study of apolipoprotein kinetics in humans.\textsuperscript{15–17} In this study, we applied these approaches to analyze the effects of ERN on Apo(a) metabolism in 8 hypertriglyceridemic patients.

### Materials and Methods

Eight nondiabetic, overweight male subjects (aged 48±12 years; body mass index, 31.2±1.8 kg/m\(^2\); waist circumference, 106±4.9 cm) with hypertriglyceridemia (triglycerides, 226±78 mg/dL) were enrolled in a randomized crossover controlled study (8 weeks/phase and a 4-week washout interval) comparing 2 g/day ERN (gradually increased from 0.5 to 2.0 g/day in the first 4 weeks to avoid flushing effects and associated with both arms with 300 mg/day of lysine acetylsalicylate) with placebo. Plasma lipids and lipoproteins, as well as lipoprotein kinetics, were assessed as described in detail in the supplemental Materials and Methods section available in the online-only Data Supplement.

### Results

Overall, ERN treatment was well tolerated and no dropout or major flushes were observed. A small and nonsignificant increase of fasting blood glucose and insulin concentrations were observed with ERN (not shown) as well as HOMA-IR index (3.8±3.7 versus 5.6±2.7, NS) Compared with placebo,

### Table. Results of Major Biochemical Measurements

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<tr>
<th>Patients</th>
<th>TG</th>
<th>TC</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>PCSK9</th>
<th>ApoA-I</th>
<th>Plasma ApoB100</th>
<th>VLDL-ApoB100</th>
<th>LDL-ApoB100</th>
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| ERN      |      |      |       |       |       |        |                |              |             |               |             |
| No. 1    | 62   | 128  | 66    | 49    | 179   | 120    | 50             | 3            | 16          | 45            | 13          |
| No. 2    | 111  | 241  | 136   | 57    | 188   | 140    | 100            | 6            | 65          | 29            | 13          |
| No. 3    | 89   | 184  | 118   | 49    | 80    | 130    | 90             | 13           | 66          | 48            | 18          |
| No. 4    | 79   | 195  | 112   | 67    | 149   | 170    | 80             | 6            | 75          | 44            | 12          |
| No. 5    | 96   | 132  | 74    | 38    | 154   | 130    | 60             | 10           | 36          | 32            | 12          |
| No. 6    | 117  | 200  | 115   | 48    | 158   | 130    | 80             | 7            | 43          | 42            | 12          |
| No. 7    | 125  | 199  | 131   | 43    | 133   | 136    | 117            | 13           | 48          | 75            | 17          |
| No. 8    | 262  | 160  | 68    | 39    | 197   | 130    | 70             | 7            | 52          | 34            | 13          |
| Mean     | 118  | 180  | 103   | 49    | 155   | 136    | 81             | 8            | 50          | 44            | 14          |
| SD       | 62   | 38   | 29    | 10    | 37    | 15     | 22             | 3            | 19          | 14            | 3           |

\(P\) value* 0.023 0.008 0.039 0.008 0.008 0.531 0.008 0.156 0.211 0.008 0.750

*Nonparametric Wilcoxon–matched pairs signed rank test.

PCSK9, ApoA-I, ApoB100, and Apo(a) plasma concentrations expressed in ng/mL, mg/dL, mg/dL, and nM, respectively. KIV repeats were assessed by the targeted liquid chromatography–tandem mass spectrometry (MS)/MS analysis of a specific peptide of the repeated domain as described in the supplemental file. ApoA indicates apolipoprotein A; ERN, extended-release nicotinic acid; HDL-C, high-density lipoprotein cholesterol (mg/dL); KIV, Kringle IV; LDL-C, low-density lipoprotein cholesterol (mg/dL); N/C, not calculated; TC, total cholesterol (mg/dL); TG, plasma triglycerides (mg/dL); and VLDL, very low-density lipoprotein.
ERN decreased plasma triglycerides by 46% (118±62 versus 221±67 mg/dL; \(P=0.023\)), total cholesterol by 16% (180±38 versus 215±49 mg/dL; \(P=0.008\)), LDL-C by 20% (103±29 versus 128±45 mg/dL; \(P=0.039\)), ApoB100 by 22% (81±22 versus 104±29 mg/dL; \(P=0.008\)), and Apo(a) by 20% (44±14 versus 54±15 mmol/L; \(P=0.008\)); Table). In contrast, ERN raised high-density lipoprotein cholesterol by 20% (49±10 versus 41±13 mg/dL; \(P=0.008\)) but did not increase apolipoprotein A-I concentrations. Plasma PCSK9 concentrations were decreased by 29% with ERN (180±53 versus 260±70 ng/mL; \(P=0.008\)); Table).

ERN affected the mean \(^2\text{H}_3\)-Leucine incorporation during the course of the tracer infusion for Apo(a) \((P<0.05\); Figure 1). Compared with placebo, ERN decreased the fractional catabolic rate (FCR) of Apo(a) by 37% \((0.36±0.19 \text{ versus } 0.58±0.28 \text{ pool/d; } P=0.008)\), whereas no significant change was observed in ApoB100- VLDL (Figure 2), and a slight increase of ApoB100-LDL FCR was shown \((+12\%; P=0.031; 1\text{-tailed})\). ERN treatment also reduced the Apo(a) production rate by 50% compared with placebo \((0.7±0.4 \text{ versus } 1.4±0.8 \text{ nmol/kg per day; } P=0.008)\) but had no significant effects on ApoB100-VLDL or ApoB100-LDL production rates (Figure 2). Apo(a) FCRs and production rates were significantly correlated \((P<0.0001; r=0.8223)\) suggesting that these 2 processes are under control of similar pathways. No significant correlations were observed between ApoB100 plasma concentrations and Apo(a) kinetic data. Similarly, no significant correlation was observed between HOMA-IR index or changes in PCSK9 plasma concentrations and Apo(a) catabolic rates.

**Discussion**

The new liquid chromatography–tandem mass spectrometry method used in this study enabled analysis of in vivo effects of ERN on Apo(a) metabolism in hypertriglyceridemic subjects. As expected, ERN significantly decreased triglycerides and Apo(a) and ApoB100 plasma concentrations and raised high-density lipoprotein cholesterol. Apo(a) production and catabolic rates were significantly reduced, while no significant changes were observed in ApoB100- VLDL kinetics. An increase in ApoB100-LDL catabolism was shown, which may be related to the significant decrease of PCSK9 plasma concentrations.

Because of high discrepancies in plasma concentrations and complexity of size determination, conventional analytical methods \(^{16,17}\) are not relevant for Apo(a) measurements. This study confirmed that enzymatic proteolysis and subsequent liquid chromatography–tandem mass spectrometry analysis of specific peptides overcome these difficulties (Materials and Methods section in the online-only Data Supplement). \(^{15,17}\) Our results are in agreement with a study using similar methods and a single compartment model to analyze Apo(a) metabolism in 3 healthy subjects \(^{16}\) and to other data reported in a using gas chromatography–mass spectrometry study and a multicompartment modeling. \(^{20}\) Together, these comparisons validated our analytic method and the mono compartmental model we have used. Compared with studies in healthy subjects without dyslipidemias, this study showed that hypertriglyceridemia does not have a strong effect on Apo(a) kinetics, although data are in near the upper limits of the reported normal range. \(^{20}\) We were also amazed to observe a slight decrease of size of Apo(a) in few subjects with ERN treatment. This was probably because of a slight change in the proportion of the 2 isoforms related to unknown effects of ERN or more likely to the tolerated precision of the liquid chromatography–tandem mass spectrometry method \((±15\%)\) and the small number of patients.

This study showed that ERN was able to significantly decrease Apo(a) production rates by 50%, consistent with in vitro studies revealing the impacts of ERN on Apo(a) hepatic production and on ApoB100 availability for Lp(a) synthesis. \(^{21}\) Nicotinic acid is already known to inhibit hepatocyte diacylglycerol acyltransferase-2, a key enzyme for triglyceride synthesis, accelerating intracellular hepatic ApoB100 degradation and decreasing hepatic ApoB100- VLDL and ApoB100-LDL secretion rates. \(^{21}\) Other studies have shown that nicotinic acid increased retention of Apo(a) at the hepatocyte surface and also directly inhibited transcription of the Apo(a) gene. \(^{1,13}\) This study did not show any association between ApoB100-LDL or...
ApoB100-VLDL and Apo(a) production rate, in agreement with a previous kinetic study of untreated healthy controls having showed that the kinetics of Apo(a)-Lp(a) were similar to ApoB100-Lp(a) but not to ApoB100-VLDL or ApoB100-LDL.\textsuperscript{20} The modification of Apo(a) production rate by ERN could thus be a consequence of an inhibition of Apo(a) transcription or a decrease of protein release as discussed above. In addition, ERN did not show significant changes on ApoB100 kinetic parameters in VLDL as previously reported by Lamon-Fava et al.\textsuperscript{22} Conversely, Fabbrini et al\textsuperscript{23} have found a significant effect of ERN to reduce VLDL-ApoB100 production rates but the different metabolic states of patients (hepatic steatosis) and the longer duration of treatment (16 weeks) could explain these differences.

Results also showed that ERN was able to significantly decrease the rate of Apo(a) catabolism by 37%, which partially compensated for the change in production rates in the liver. Although catabolic mechanisms of Lp(a) are poorly understood, kidneys seem to play central roles involving binding with megalin/glycoprotein 330.\textsuperscript{24} In patients with advanced chronic kidney diseases, elevated plasma Lp(a) concentrations were observed,\textsuperscript{25} which were related to decreases in Apo(a) and ApoB100 Lp(a) catabolic rates.\textsuperscript{26} It has also been shown that, in patients with chronic kidney diseases, ENR has reduced efficacy.\textsuperscript{27}

In addition to the kidney, other mechanisms are likely to occur in the liver. Indeed, Lp(a) metabolism has been linked to the scavenger receptor class B type 1,\textsuperscript{28} the low-density lipoprotein receptor–related protein 1, the very low-density lipoprotein receptor, plasminogen receptors, and megalin/glycoprotein 330.\textsuperscript{24} The role of the LDL receptor on Lp(a) catabolism is controversial. A recent study in HepG2 cells provided evidence that the LDL receptor is implicated in Lp(a) catabolism modulated by PCSK9.\textsuperscript{29} Our data do not support this finding as we showed a significant decrease in ApoB100 plasma concentrations (−22%) probably related to the increase of ApoB100-LDL catabolism (+12%) possibly as a consequence of the decrease in PCSK9 plasma concentrations known to occur with ERN.\textsuperscript{30,31} The increase of ApoB100-LDL FCR suggested an enhanced the LDL receptor activity and an increase of Apo(a) catabolism was expected from the HepG2 cell experiment.\textsuperscript{29} We have observed just the opposite but it is possible that the role of the LDL receptor in Lp(a) catabolism could have been overshadowed by changes in other Lp(a) catabolic pathways related to ERN as we measured the total in vivo disappearance. In addition, no significant relationship was observed between changes of Apo(a) FCR and PCSK9 plasma concentrations ($P=0.2436; r=0.4668$), which do not support a central role of PCSK9 in Apo(a) catabolism.

This study has some limitations. To follow the guidelines of ERN prescription, only patients with hypertriglyceridemia were selected. Although unlikely, it is not possible to rule out that different results could have been observed in lean normolipidemic subjects. It is also difficult to anticipate the mechanisms involved in patients with elevated baseline Lp(a) and the effect of ERN in these individual. We were not able to get a good recovery of Lp(a) in the ApoB100 containing particles. Then, we only present Apo(a) kinetics as a whole and not the fine tuning and exchanges of Lp(a) related to measured activities of lipases or transfer proteins. However, this simple approach allows to get new information on the mechanisms involved in the decrease of plasma Lp(a) related to ERN treatment and could initiate new researches. Finally, this study was performed in men with no statin treatment and it is not known if the sex or an added treatment could have changed the results.

In conclusion, decreased plasma Lp(a) in ERN-treated patients was related to a decrease of Apo(a) production rate, which was partially compensated by decreased catabolism. The regulatory mechanisms involved in changes of both production and catabolism of Apo(a) by ERN seem to be connected. No cardiovascular benefits of ERN were reported in

![Figure 2. Individual and mean±SEM changes in kinetic parameters of Apo(a) and ApoB100. FCR indicates fractional catabolic rate; and PR, production rate. *$P<0.05$, **$P<0.01$ (nonparametric Wilcoxon-signed rank test).](http://atvb.ahajournals.org/)

\begin{itemize}
\item ApoB100-VLDL and Apo(a) production rate, in agreement with a previous kinetic study of untreated healthy controls having showed that the kinetics of Apo(a)-Lp(a) were similar to ApoB100-Lp(a) but not to ApoB100-VLDL or ApoB100-LDL.\textsuperscript{20}
\item ERN did not show significant changes on ApoB100 kinetic parameters in VLDL as previously reported by Lamon-Fava et al.\textsuperscript{22}
\item Fabbrini et al\textsuperscript{23} have found a significant effect of ERN to reduce VLDL-ApoB100 production rates.
\item The role of the LDL receptor on Lp(a) catabolism is controversial.
\item Our data do not support this finding.
\item ENR has reduced efficacy.
\item This study has some limitations.
\item In conclusion, decreased plasma Lp(a) in ERN-treated patients was related to a decrease of Apo(a) production rate, which was partially compensated by decreased catabolism. The regulatory mechanisms involved in changes of both production and catabolism of Apo(a) by ERN seem to be connected.
\end{itemize}
recent cardiovascular outcome trials but with a decrease of plasma Lp(a) of only 20%.32–37 A better understanding of the mechanisms responsible for decreased Lp(a) catabolism associated with ERN treatment could help the development of new derived niacin treatments targeting only the production rate leading to a more effective therapy for lowering Lp(a) plasma concentrations.

Acknowledgments
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Disclosures
None.

References
Apolipoprotein (a) is a major component of lipoprotein (a) and has a genetically controlled, highly polymorphic size strongly associated with risk of coronary heart disease. Although some treatments, such as extended-release nicotinic acid, lower lipoprotein (a) plasma concentrations, the exact mechanisms underlying the reductions remain unclear. A liquid chromatography–tandem mass spectrometry method was recently developed for apolipoprotein (a) measurements. This method was used here to study apolipoprotein (a) kinetics in 8 hypertriglyceridemic patients treated with extended-release nicotinic acid. Treatment decreased apolipoprotein (a) plasma concentrations by 20% and decreased production rates by 50%. These changes were partially compensated by decreased catabolism (−37% compared with placebo). Apolipoprotein B100 and proprotein convertase subtilisin/kexin type 9 plasma concentrations were also decreased by treatment, but no correlation was found with apolipoprotein (a) kinetic parameters. Therefore, extended-release nicotinic acid has impacts on lipoprotein (a) levels and apolipoprotein (a) synthesis and catabolism.
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SUPPLEMENTAL FILE

MATERIALS AND METHODS

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MATERIAL AND METHODS

Patients

Patients that met inclusion criteria (male, age 18 to 65 years, plasma triglyceride concentration 150 to 400 mg/dL, high-density lipoprotein cholesterol (HDL-C) below 60 mg/dL, waist circumference > 94 cm and body mass index 27 to 35 kg/m²) were included in a double blind, placebo controlled cross-over 8 week study. These criteria were selected according to the European definition of the metabolic syndrome from the International Diabetes Federation (1) and also according to the ERN guidelines for prescription (2).

Patients diagnosed with cancer, diabetes mellitus, hepatic, renal, or digestive disorders, and hypertension and those that received chronic medical treatment that interfered with lipid metabolism were excluded from the study.

Each subject received extended-release nicotinic acid (ERN, Niaspan®, Merck Clevenot, France) and placebo in a random order for eight weeks. A two-week interval was included between the two treatments. To minimize blinding effects, the daily dose of ERN was gradually increased from 0.5 to 2.0 g/day in the first four weeks, then kept constant at 2.0 g/day during the last four weeks and associated in both arms with 300 mg/day of lysine acetylsalicylate). Regular phone calls and intermediate visit (4 weeks) were organized to control for treatment compliance. At the end of each treatment phase, ERN or placebo were given after an overnight fast, and subjects received a 10 µM/kg bolus injection of [5,5,5-²H₃]-L-Leucine immediately followed by constant infusion of [5,5,5-²H₃]-L-Leucine (10 µM/kg/h) for 14 hours. Subjects were only allowed to drink water until the end of the tracer infusion.

Blood samples were collected at 0, 15, 30, 45, 60, 90, 120, 150, and 180 minutes then at 4, 6, 8, 10, 12 and 14 hours. 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).

Sample collection and preparation

Blood samples were collected in EDTA tubes (Venoject, Paris, France), and plasma was separated by centrifugation at 4 °C for 30 minutes and stored at -80 °C until analysis.

Lipoproteins fractions, including very low-density lipoprotein (VLDL, <1.006 g/mL), intermediate density lipoprotein (IDL, 1.006 to 1.019 g/mL), low-density lipoprotein (LDL, 1.019 to 1.063 g/mL) and high-density lipoprotein (HDL, 1.063 to 1.210 g/mL), were separated by density gradient ultracentrifugation as described previously (3, 4) and stored at -80 °C until analysis.

Chemicals

UPLC/MS-grade acetonitrile, water, and 99% formic acid were purchased from Biosolve (Valkenswaard, Netherlands). Ammonium bicarbonate (AB), 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 peptides LFLEPTQADIALLK, [5,5,5-²H₃]L-LFLEPTQADIALLK, LFLEPTQADIALL-[¹³C₆¹⁵N₂]K, GTYSTTVTGR, and GTYSTTVTG-[¹³C₆¹⁵N₂]JR were purchased from Thermo Scientific Biopolymers (Einsteinstrasse, Germany).

Measures of plasma concentrations of the lipid and glucose parameters

Cholesterol and triglyceride concentrations were measured using enzymatic test kits from Boehringer Mannheim GmbH (Mannheim, Germany). ApoA-I concentrations were measured by immunonephelometry (Behring, Rueil Malmaison, France), and ApoB100 concentrations were obtained using selective precipitation and mass spectrometry (5). Plasma PCSK9 concentrations were measured by ELISA (R&D Systems, Lille, France). Fasting blood glucose and insulin concentrations were measured as previously reported as well as the HOMA-IR index calculation (6).

Measurement of ApoB100 Leucine enrichment
Isolation and measurement of Leucine enrichment in ApoB100 were described previously (3). Briefly, ApoB100-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 heptaffluorobutyric acid), and analyzed by gas chromatography-mass spectrometry to determine [5,5,5-^3H_3]-L-Leucine enrichment.

Selection of proteotypic Apo(a) peptides
Apo(a) sequences were Blast searched using the UNIPROT tool (www.uniprot.org), and theoretical proteotypic peptides were searched using the free software peptide mass calculator (http://web.expasy.org/peptide_mass). Peptide candidates were selected to maximize sensitivity, specificity, and stability. Peptides containing methionine and cysteine were not considered due to potential oxidation. As described by Lassman et al. (7) and Zhou et al. (8), LFLEPTQADIALLLK was selected for Apo(a) quantification and kinetic measurements, and GTYSTTVTGR was selected for Apo(a) size characterization. The LFLEPTQADIALLLK and GTYSTTVTGR peptides are located in the Peptidase S1 and kringle IV2 domains, respectively.

Sample preparation for Apo(a) quantitation and size characterization in plasma
LFLEPTQADIALLLK, LFLEPTQADIALLL-[^13C_6-^15N_2]K, GTYSTTVTGR, and GTYSTTVTGR-[^13C_6-^15N_2]R synthetic peptides were dissolved in a water/acetonitrile mixture (50/50, v/v) containing 0.1% formic acid at a concentration of 100 µM and were stored at -20°C in aliquots of 20 µL. Stock solutions of LFLEPTQADIALLLK and GTYSTTVTGR were then mixed and diluted in 0.5% SDC to produce seven 10X standard solutions ranging from 5.0 to 0.05 µM and 20.0 to 0.2 µM for LFLEPTQADIALLLK and GTYSTTVTGR, respectively.

Plasma samples (10 µL) were mixed and diluted with 15 µL of 0.5% SDC. LFLEPTQADIALLLK-[^13C_6-^15N_2]K and GTYSTTVTGR-[^13C_6-^15N_2]R were used as internal standards (ISs). Ten microliters (10 µL) of each IS stock solution were extemporaneously added to 9,980 µL of a reduction buffer (pH 8) containing 55 mM AB, 0.5% SDC, and 5.5 mM DTT. Twenty microliters of 10X standard solutions and diluted plasma samples were added to 180 µL of reduction buffer containing both ISs. Final concentrations of AB, DTT, and ISs were 50 mM, 5 mM, and 180 nM, respectively. The final concentrations of standard solutions were from 500 to 5 nM and from 2000 to 20 nM for LFLEPTQADIALLLK and GTYSTTVTGR, respectively. Samples were reduced for 30 minutes at 60 °C then alkylated with 2 µL of fresh IA solution (1 M in 1 M NaOH) for 60 minutes at room temperature (protected from light). Samples were digested overnight with 10 µL of trypsin solution (0.1 mg/ml in 1 mM HCl), and 10 µL of 20% formic acid were added to stop the reaction and to precipitate the SDC. Samples were centrifuged for 15 minutes at 20,000 g, 4 °C and 180 µL of supernatants were transferred to LC vials for LC-MS/MS analyses.

Sample preparation for Apo(a) kinetic measurements in lipoprotein fractions
To recover the major Lp(a) particles, 100 µL of LDL and HDL lipoprotein fractions were combined (7) and desalted and concentrated with 3 mL of 50 mM AB buffer (pH 8) and a 5-kDa molecular weight cut-off filter. Concentrated samples (100 µL) were mixed with 88 µL of 50 mM AB buffer (pH 8), 10 µL of 10% SDC, and 2 µL of 500 mM DTT and reduced at 60 °C for 30 minutes. Samples were then treated as described in the previous section.

LFLEPTQADIALLLK and [5,5,5-^3H_3]-L-FLEPTQADIALLLK synthetic peptides were dissolved in a water/acetonitrile mixture (50/50, v/v) containing 0.1% formic acid to reach a final concentration of 500 nM. To evaluate accuracy, labeled peptide solution was diluted in unlabeled peptide solutions to reach theoretical enrichments ranging from 0 to 5% (0, 0.1, 0.25, 0.5, 1.0, 2.0, and 5.0%, n = 6/point). Control samples were prepared with the same method at 2% enrichment and for concentrations of 500, 250, 100, 50, 20 and 10 nM (n = 6/point).

Analytical parameters
Analyses were performed on an LC-MS/MS system consisting of a Xevo® Triple-Quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface and an Acquity H-Class® UPLC™ device (Waters Corporation, Milford, MA, USA). Data acquisition and analyses were performed using MassLynx® and TargetLynx® software, respectively (version 4.1, Waters Corporation, Milford, MA, USA). Labeled and unlabeled peptides were separated on an Acquity® BEH C18 column (2.1 × 100 mm, 1.7 μm, 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 μL/min. Mobile phase B was linearly increased from 1 to 50% for 5 minutes, kept constant for 1 min, returned to the initial condition in 1 min, and kept constant for 1 min before the next injection. Ten microliters of each sample were injected into the LC column. Labeled and unlabeled peptides were then detected by the mass spectrometer equipped with an ESI interface operating in the positive ion mode. The multiple reaction monitoring (MRM) mode was applied for MS/MS detection. Selected MRM transitions, cone voltages, and collision energies are described in Supplemental Table I (supplemental material). Note that each precursor ion was detected as a doubly charged ion leading to the formation of singly charged product ions (y10+ for LFLEPTQADIALLK and labeled analogues and y7+ for GTYSTTVTGR and labeled analogue).

Apo(a) data management

Chromatographic peak area ratios between target peptides and their respective ISs constituted detector responses. Standard solutions were used to plot calibration curves for quantification of both peptides (Supplemental Figure I in supplemental material). A linear regression model was used for peptide quantification (7 concentration levels, n = 6), and the linearity of the method was confirmed (r² at 0.9988 and 0.9998 for LFLEPTQADIALLK and GTYSTTVTGR, respectively). Each plasma sample was assayed three times, and the coefficients of variation did not exceed 6.8% and 12.7% for LFLEPTQADIALLK and GTYSTTVTGR, respectively. As targeted peptide LFLEPTQADIALLK is unique in Apo(a), 1 mole of Apo(a) was assumed to be equal to 1 mole of this peptide. As the target peptide GTYSTTVTGR is repeated in the kringle IV1, IV2, IV3 and IV5 regions but not in the IV4, IV6, IV7, IV8, IV9 and IV10 regions, the total number of kringle IV was equal to (((GTYSTTVTGR)/[LFLEPTQADIALLK]) + 6) taking into account that the result expressed the average Apo(a) size of the two circulating isoforms.

For Apo(a) enrichment measurements, M3/M0 ratios were calculated using chromatographic peak areas; M3 corresponded to the [5,5,5-2H3]-L-Leucine labeled peptide and M0 to the unlabeled peptide. As the selected peptide contains 4 Leucines and 4 isotopomers of M3 can be formed (Supplemental Table I), the sum of MRM transitions used for the M3 detection allowed detection of all the isotopomers. Thus, M3/M0 ratios measured in biological samples were corrected by dividing the primary result by 4, as described by Brunengraber et al. (9).

Correction was not applied in standard enrichment samples prepared with one standard isotopomer (Supplemental Figure I). Agreement was observed at 500 nM between the measured and expected ratio of [5,5,5-2H3]-L-Leucine labeled peptide (M3) and unlabeled peptide (M0) over seven enrichment levels (n = 6). The slope and r² of the linear regression were 0.9987 and 1.06, respectively. To gain confidence in the ratio measurements, a theoretical enrichment at 2% was performed over six distinct concentrations of LFLEPTQADIALLK (n = 6). No significant variation was observed from 10 to 500 nM. The coefficients of variation calculated over the six replicates did not exceed 8.3%, and the mean difference calculated between theoretical and measured enrichments was below 6.0%.

Apo(a) and ApoB100 kinetic modeling

Apo(a) fractional catabolic rate (FCR) was estimated on the 14 hour samples and using the SAAM II modeling program (Epsilon Group, Charlottesville, VA, USA) and fitting a mono exponential curve (mono compartmental model), as suggested for proteins with slow turnover rates (8). Apo(a) was assumed to have the corresponding VLDL-ApoB100 leucine plateau enrichment as a precursor pool. According to this steady state model, the FCR was
considered equivalent to the fractional synthetic rate (FSR). Kinetic parameters of VLDL and LDL ApoB100 were calculated from the same plasma samples and using a three-compartmental model as described previously for a 14 hours of a tracer constant infusion (3). Individual kinetic data are shown in supplemental Table II (supplemental material).

Statistical analyses
Statistical analyses were performed with GraphPad Prism software (version 6.0, GraphPad Software Inc., La Jolla, CA, USA) using the nonparametric Wilcoxon matched-pairs signed rank test. Results are expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Differences between ERN and placebo treatment were considered statistically significant at p <0.05 (two-tailed or otherwise specified).

REFERENCES
SUPPLEMENTAL MATERIAL

Effects of extended-release nicotinic acid on apolipoprotein (a) kinetics in hypertriglyceridemic patients

Mikaël Croyal, Khadija Ouguerram, Maxime Passard, Véronique Ferchaud-Roucher, Maud Chétiveaux, Stéphanie Billon-Crossouard, Gilles Lambert, Michel Krempf and Estelle Nobécourt.
### Supplemental Table I. Mass spectrometry parameters for peptide detection.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Fragment</th>
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<tr>
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RT: retention time
**Supplemental Table II. Individual kinetic parameters of Apo(a) and ApoB100.**

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<tr>
<td>SD</td>
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<td>0.4</td>
<td>2.53</td>
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</table>

**p value** 0.008 0.008 0.742 0.313 0.031* 0.844

FCR: fractional catabolic rate (pool/day), PR: production rate (nmol/kg/day for Apo(a), and mg/kg/day for ApoB100), SD: standard deviation.

Nonparametric Wilcoxon matched-pairs signed rank test, *one-tailed
Supplemental Figure I. (A) Calibration curves used for Apo(a) quantification. (B) Calibration curves used for Apo(a) average size characterization. (C) Linearity of M3/M0 measurements at 500 nM for theoretical enrichments ranging from 0.1 to 5.0%. (D) Accuracy of enrichment assay (2%) for Apo(a) concentrations ranging from 10 to 500 nM. Values are mean ± SD. M3: [5,5,5-2H_3]-L-Leucine labeled peptide, M0: unlabeled peptide.