Abstract

Approximately 60% of patients reaching low-density lipoprotein (LDL) cholesterol (LDLC) therapeutic goal under lipid-lowering therapy remain at high cardiovascular risk.1 Such patients are often characterized by a low-hi-density lipoprotein (HDL) cholesterol (HDLCh) phenotype and high circulating levels of triglyceride (TG)-rich lipoproteins (TRL). Designated as a broad spectrum lipid-lowering agent, niacin was the first therapy used to reduce total cholesterol, LDLc, and TG and to increase HDLCh levels.2 In this context, adding niacin to a background of statin therapy was a promising concept to reduce residual cardiovascular risk. However, 2 major clinical trials, The Atherothrombosis Intervention in Metabolic Syndrome with low HDL/high TG: Impact on Global Health Outcomes (AIM-HIGH) and Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE), failed to demonstrate the efficacy of niacin to further reduce occurrence of cardiovascular events in high cardiovascular-risk patients receiving a statin therapy.3-5 Despite observational studies indicating an inverse relationship between HDLC levels and cardiovascular risk,6 recent outcome trials on cholesteryl ester transfer protein (CETP) inhibition7 along with a study on human genetics8...
phenotype are characterized by an exacerbated postprandial text, it has been reported that patients displaying a low HDL fractionation and accumulation of TRL is associated with an acceleration of RCT.12,13 Indeed, it is important to consider that the capacity of HDL to mediate cholesterol efflux represents a strong predictor of the presence and the extend of atherosclerosis.14 Equally, an inverse association between the prevalence of coronary disease and HDL efflux capacity has been observed; however, HDL-mediated efflux was paradoxically associated with increases in prospective risk of myocardial infarction, stroke, and death.15 These observations suggest that it is important to consider the overall efficacy of RCT pathway, including not only macrophase cholesterol efflux, but also the return of cholesterol to the liver and the ultimate excretion of cholesterol from the body.

The efficacy of RCT pathway can be influenced by several factors. Postprandial lipemia, characterized by a transient production and accumulation of TRL, is associated with an acceleration of RCT.12,13 Indeed, it is important to consider that intravascular metabolism of both TRL and HDL is intimately intricate: (1) CETP-mediated neutral lipid hetero-exchange between TRL and HDL induces the formation of CE-rich TRL remnants and TG-rich HDL particles and (2) the transfer of phospholipids and cholesterol from lipid surface of TRL to HDL allows formation of mature HDL particles.14 In this context, it has been reported that patients displaying a low HDL phenotype are characterized by an exacerbated postprandial hypertriglyceridemia,15 indicating that reduced HDL levels directly impact intravascular metabolism of TRL particles. In such patients, postprandial phase is associated with an increase in cholesterol efflux from macrophages as a result of CETP-mediated intravascular HDL remodeling and liberation of lipid poor/lipid free apolipoprotein (apo) AI.16 However, enhanced CETP-mediated CE transfer from HDL to postprandial TRL induces formation and accumulation of CE-enriched lipoprotein remnants that represent preferential precursors of atherogenic small dense LDL particles, displaying a reduced affinity for the hepatic LDLR.17 Equally, HDL particles generated during postprandial phase have been shown to display a reduced ability to deliver CE to the liver.18 Taken together, these observations indicate that despite improvement of cholesterol efflux from macrophage, postprandial lipemia is associated with a global reduction of RCT efficacy because the ultimate return of cholesterol to the liver, directly or indirectly, is reduced in patients displaying a low HDL phenotype.

Niacin has long been known to efficiently reduce the coexisting lipid triad consisting of TRL, TRL remnants, and small dense LDL particles in hyperlipidemic patients.18 In addition, it has been reported that extended-release niacin (ERN) given in a single dose 1 hour before oral fat tolerance test significantly reduces postprandial TG levels in healthy volunteers.19 The objective of the present study was to evaluate the impact of ERN/laropiprant (LRPT) on the overall efficacy of RCT pathway after ingestion of a typical western meal in dyslipidemic patients at high cardiovascular risk on a background of statin treatment. RCT pathway was evaluated through an integrated functional approach by measuring its major steps, including the initial macrophase cholesterol efflux, CETP-mediated CE transfer from HDL to apoB-containing lipoprotein, and in vivo hepatic HDL-CE uptake, as well as in vivo HDL-mediated RCT to feces.

Materials and Methods
Detailed information is available in Materials and Methods in the online-only Data Supplement.

Results
Impact of ERN/LRPT Therapy on Fasting Plasma Lipid and Apolipoprotein Levels
Before initiation of ERN/LRPT therapy, dyslipidemic patients received statins (20–40 mg/d) during 4 weeks to stabilize their plasma lipid levels within the normal range (Table). After administration of ERN/LRPT on background of statins during 12 weeks, significant reductions in plasma levels of TGs (−26%; P<0.02), total cholesterol (−12%; P<0.02), LDLC (−21%; P<0.002), and apoB (−21%; P<0.001) were observed. Concomitantly, a significant elevation in HDLC levels (+17%; P=0.02) were observed; however, those of both apoAI and apoAII were only slightly and not significantly modified by ERN/LRPT therapy. Note that the ERN/LRPT-induced changes in lipid parameters presently reported are entirely consistent with known effects of niacin or with those previously reported for ERN/LRPT in patients with dyslipidemia.20 Interestingly, plasma levels of apoE were reduced by 19% (P<0.03) after ERN/LRPT, whereas those of apoCII and apoCIII were not significantly affected. Taken together, these observations indicate that even under stable statin therapy, administration of ERN/LRPT in dyslipidemic patients allow the establishment of an anti-atherogenic plasma lipid profile as shown by the reduction in total cholesterol to HDLC ratio (−23%; P<0.01).

Impact of ERN/LRPT Therapy on Postprandial Lipoprotein Subspecies
Combination therapy with niacin–laropiprant significantly reduced postprandial hypertriglyceridemia after consumption
of a typical solid mixed meal as shown by significant reduction of iAUC of TG (−53%; P < 0.02; Figure 1A). Such variations predominantly reflect postprandial changes in large TRL sub-subs, that is, chylomicrons (iAUC-CM-TG: −53%; P < 0.004; Figure 1B) and VLDL-1 (area under curve [AUC]-VLDL-1-TG: −44%; P < 0.0001; Figure 1C). Interestingly, ERN/LRPT treatment abolished the postprandial elevation of VLDL-1-TG levels. In addition, treatment with ERN/LRPT significantly reduced circulating levels of VLDL-2 particles; however, no significant postprandial variation in VLDL-2 levels was observed (Figure 1C). Taken together, our results revealed that ERN/LRPT reduces the magnitude of postprandial TRL levels.

After ERN/LRPT therapy, we observed a reduction in AUC for plasma LDLc levels (−16%; P < 0.02, Figure 1A in the online-only Data Supplement). By contrast, the AUC for plasma HDLc levels was significantly increased by ERN/LRPT therapy (+13%; P < 0.05) as a result of a specific increment (+17%; P < 0.05) in the AUC for large HDL2 subfraction (Figure 1B in the online-only Data Supplement). Analysis of individual HDL subspecies revealed that ERN/LRPT-induced elevation in postprandial plasma levels of HDLC exclusively results from a specific increment in circulating levels of large HDL2b subfraction (+17%; P < 0.05). Note that postprandial variations in circulating levels of both LDLC and HDLC are entirely consistent with those reported in earlier studies. Independently of ERN/LRPT treatment, no significant impact of postprandial phase was observed on plasma levels of apoB, apoAI, and apoAII levels (Figure 1A and IB in the online-only Data Supplement).

Before ERN/LRPT treatment, postprandial phase was associated with a significant increment in circulating apoE levels, reaching a peak 4 hour after meal intake. Interestingly, such postprandial increase in apoE levels was abolished after ERN/LRPT treatment (Figure 2A). Equally, before ERN/LRPT treatment, test meal consumption had no impact on apoCII levels, which remain constant throughout the postprandial phase. Interestingly, ERN/LRPT induced a significant postprandial elevation in plasma levels of apoCII (Figure 2B), which occurred concomitantly with a reduction in apoCIII levels (Figure 2C). Because apoCII and apoCIII, respectively, represent activator and inhibitor of lipoprotein lipase, these observations suggest that intravascular catabolism of TRL particles is accelerated after ERN/LRPT treatment.

Impact of ERN/LRPT on Key Steps of Reverse Cholesterol Transport Pathway During Postprandial Phase

We observed a significant impact of niacin–laropiprant on postprandial plasma cholesterol efflux capacity from human macrophages (−9.3%; P < 0.04; Figure 3A) and via ATP-binding cassette A1 (ABCA1) pathway (−8.5%; P < 0.05; Figure 3B). Interestingly, opposite variations were observed on scavenger receptor class B type 1 (SR-B1) and ATP-binding cassette G1 (ABCG1) pathways because ERN/LRPT induced a slight but significant increase in AUC of SR-B1-dependent plasma efflux capacity (+5.5%; P < 0.05; Figure 3C) and of ABCG1-dependent plasma efflux capacity (+5.2%; P < 0.05; Figure 3D). Efflux experiments have been equally performed using apoB-depleted plasma as cholesterol efflux acceptors (Figure II in the online-only Data Supplement). ERN/LRPT treatment was without any significant effect on cholesterol efflux to apoB-depleted plasma evaluated on human THP-1 macrophages or on CHO cells over expressing ABCA1. By contrast, SR-B1-dependent cholesterol efflux to apoB-depleted plasma was significantly increased after ERN/LRPT therapy as a result of potentially niacin-induced elevation in circulating levels of large HDL particles. There was no significant impact of ERN/LRPT on the capacity of isolated postprandial HDL subspecies to mediate cholesterol efflux either from human macrophages or through specific efflux pathway (Figure III in the online-only Data Supplement). Taken together, our observations indicate that the increase in plasma cholesterol efflux capacity from human macrophages primarily involves the SR-B1 pathway and is mainly related to an increase in the quantity of HDL with no modification of their intrinsic efflux capacity.

Before ERN/LRPT therapy, we observed that endogenous CETP activity increased significantly during postprandial state and peaked 6 hours after meal intake (+22%; P < 0.0001).

Table. Fasting Plasma Lipids and Apolipoprotein Levels in Dyslipidemic Patients Before and After ERN/LRPT Treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Statin Therapy Alone</th>
<th>Statin Therapy + ERN/LRPT</th>
<th>P Value</th>
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<tr>
<td>Age, y</td>
<td>64±9</td>
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<tr>
<td>Sex, M/F</td>
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<td>Body mass index, kg/m²</td>
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<td>Waist circumference, cm</td>
<td>93±12</td>
<td>90±11</td>
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<td>Systolic blood pressure, mm Hg</td>
<td>131±10</td>
<td>135±12</td>
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<tr>
<td>Diastolic blood pressure, mm Hg</td>
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<td>77±5</td>
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<tr>
<td>Cholesterol, mg/dL</td>
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</tr>
<tr>
<td>Total</td>
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<tr>
<td>LDL</td>
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<td>Apolipoprotein, mg/dL</td>
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<tr>
<td>A-II</td>
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<tr>
<td>B</td>
<td>96±19</td>
<td>76±23</td>
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<tr>
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<td>8.69±2.74</td>
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After a 4-week diet/statin run-in phase, patients received in addition to statin therapy, ERN/LRPT 1 g/20 mg once daily for 4 wk and 7 g/40 mg once daily for 8 additional weeks. APoA1 indicates apolipoprotein A1; ERN/LRPT, extended-release niacin/laropiprant; HDL, high-density lipoprotein; HDLC, HDL cholesterol; LDL, low-density lipoprotein; OGTT, oral glucose tolerance test; and TC, total cholesterol. Values are mean±SD.
Remarkably, such postprandial increase was abolished after ERN/LRPT treatment (Figure 3E). These observations indicate that ERN/LRPT treatment significantly reduced postprandial endogenous CETP-dependent CE transfer from HDL to apoB-containing lipoproteins, including TRL particles (AUC for CETP, 12.6%; \( P < 0.02 \) versus before ERN/LRPT treatment). In addition, after ERN/LRPT treatment, we observed a significant reduction in plasma CETP concentrations (−15%; \( P < 0.02 \)). However, we did not observe any significant variation in plasma CETP levels throughout the postprandial phase as previously reported in studies using a test meal, including a similar amount of cholesterol.24,25

Figure 1. Postprandial time course in response to ingestion of a test meal (87 g fat, 5 g protein, 19 g carbohydrates, including 152 mg of cholesterol; total energy 875 kcal) in dyslipidemic patients before (●) and after (○) 12 weeks of extended-release niacin/laropiprant (ERN/LRPT) therapy on plasma levels of triglycerides (TG; A), chylomicrons-TG (B), VLDL1-TG and VLDL2-TG (C). In all sections are represented the histograms of area under the curve (AUC) and iAUC before (closed bars) and after (open bars) treatment. Values are mean±SEM. *\( P < 0.001 \) vs before ERN/LRPT treatment.

Figure 2. Line plots showing plasma concentrations of apolipoproteins: ApoE (A), ApoCII (B), and ApoCIII (C) determined throughout the postprandial phase in patients (n=10) before (●) and after (○) 12 weeks of extended-release niacin/laropiprant (ERN/LRPT) treatment.
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The impact of ERN/LRPT treatment on the capacity of postprandial HDL particles to deliver CE to hepatic cells was evaluated using HepG2 cells (Figure 3F). Before ERN/LRPT therapy, we observed that HDL particles isolated 6 and 8 hours after meal intake displayed a significant reduction in their capacity to deliver CE to hepatic cells (−18%; P<0.03 and −17%; P<0.04, 6 and 8 hours, respectively) as compared with those isolated before meal intake. By contrast, after ERN/LRPT treatment, opposite postprandial changes were observed. Indeed, the capacity of postprandial HDL particles showed a progressive increase in their capacity to deliver CE to hepatic cells (≤8 hours postprandially (+26%; P<0.005) as compared with those isolated before meal intake. By contrast, after ERN/LRPT treatment, opposite postprandial changes were observed. Indeed, the capacity of postprandial HDL particles showed a progressive increase in their capacity to deliver CE to hepatic cells (≤8 hours postprandially (+26%; P<0.005) as compared with those isolated before meal intake. Taken together, these observations indicate that 6 and 8 hours after meal intake, HDL particles isolated from patients receiving ERN/LRPT displayed an enhanced capacity to deliver CE to hepatic cells as compared with their equivalent counterpart isolated from patients not receiving ERN/LRPT (+22.6%; P<0.009 and +39.3%; P<0.0005 at 6 and 8 hours, respectively).

Niacin Improves the Capacity of Postprandial HDL to Promote RCT to Feces In Vivo

Similar to our current observations in dyslipidemic patients, evaluation of ERN/LRPT impact on RCT efficiency in human ApoB/CETP double transgenic mice fed with chow diet supplemented with 1% niacin during an 8-week period showed significant reductions in plasma concentrations of total cholesterol (−36%; P<0.0005), triglycerides (−42%; P<0.02), and apoB (−44%; P<0.03) when compared with ApoB/CETP double transgenic mice fed with chow diet (Figure 4A–4C). Concomitantly, we observed a significant increase in HDLC levels (+11%; P<0.05) in mice receiving niacin (Figure 4D). In addition, human ApoB/CETP double transgenic mice fed with chow diet supplemented with niacin displayed a significant reduction in plasma CETP activity (−30%; P<0.05; Figure 4E) as well as in the capacity of plasma to stimulate cholesterol efflux from human THP-1 macrophages (−11%; P<0.05; Figure 4F).

The capacity of postprandial HDL particles isolated from dyslipidemic patients before and after ERN/LRPT treatment to promote RCT to feces was evaluated in vivo in human ApoB/CETP double transgenic mice fed ad libidum a normal chow diet supplemented with 1% niacin during an 8-week period. After injection, plasma clearance of radioactivity was similar for labeled HDL particles isolated from both treated and untreated patients at 0 and 8 hours after meal intake. In vivo liver uptake of radioactive tracer derived from postprandial HDL particles from untreated patients was significantly reduced (P<0.05) as compared with HDL particles isolated before meal intake. By contrast, after

Figure 3. Effects of extended-release niacin/laropiprant (ERN/LRPT) therapy on major steps of reverse cholesterol transport (RCT). Line plots showing the capacity of postprandial plasma to mediate cellular-free cholesterol efflux from THP-1 (A), and via ATP-binding cassette A1 (ABCA1; B), scavenger receptor class B type 1 (SR-BI; C), and ATP-binding cassette G1 (ABCG1; D). Cholesterol efflux from cells was determined after a 4 h incubation in the presence of 40-fold diluted plasma from 10 patients before (●) and after ERN/LRPT treatment (○). F, The postprandial time course of plasma endogenous cholesteryl ester transfer protein (CETP) activity before (●) and after (○) ERN/LRPT therapy. Line plots in F are showing the in vitro capacity of postprandial high-density lipoprotein (HDL) particles isolated from patients (n=10) before (closed lines) and after (open circles) ERN/LRPT therapy to deliver cholesteryl esters to HepG2 cells. Graphs showing AUC for cellular cholesterol efflux and CETP activity before (closed bars) and after (open bars) ERN/LRPT therapy are presented. Values are means±SEM. *P<0.05 vs before ERN/LRPT treatment.
ERN/LRPT treatment, we observed an increased ($P<0.05$) in vivo liver uptake of postprandial HDL particles as compared with HDL particles isolated before meal intake. Similarly, quantification of radioactivity excreted into feces revealed that postprandial HDL particles isolated from patients before ERN/LRPT treatment displayed a significant reduced capacity ($P<0.05$) for HDL-mediated RCT to feces. By contrast, the capacity of postprandial HDL isolated after ERN/LRPT treatment for HDL-mediated RCT to feces was enhanced or at least maintained, resulting in a significant 2-fold ($P<0.0003$) increase in the capacity of postprandial HDL particles for HDL-mediated RCT to feces as compared with their counterpart isolated before ERN/LRPT treatment.

To identify potential mechanism underlying the impact of niacin on RCT pathway efficacy, we evaluated both liver and intestine expression of various genes involved in lipid metabolism. We presently observed that niacin consumption reduced intestine expression of P2Y13 ($−26\%$; $P<0.001$) and ACAT2 ($−58\%$; $P<0.001$) and increased those of ABCA1 ($+74\%$; $P<0.003$) in human ApoB/CETP double transgenic mice (Figure 6A). Equally, niacin consumption reduced liver expression of human CETP gene ($−37\%$; $P=0.005$), P2Y13 ($−26\%$; $P<0.02$), and ABCG1 ($−37\%$; $P<0.002$) in human ApoB/CETP double transgenic mice model (Figure 6B).

**Discussion**

Here, we report that in patients at high cardiovascular risk under current statin therapy, ERN/LRPT efficiently attenuates atherogenic postprandial hypertriglyceridemia by decreasing circulating levels of TRL and improves overall RCT efficacy, thus favoring cholesterol elimination from the body.

Herein, we observed that dyslipidemic patients treated with statins displayed an exaggerated postprandial response after meal intake. ERN/LRPT reduced such postprandial hypertriglyceridemic response by almost 50%, showing an additional beneficial effect of niacin over statin treatment. This observation is in good accordance with a previous study showing that ERN lowered postprandial triglyceridemia in hypertriglyceridemic response by almost 50%, showing an exaggerated postprandial response after meal intake. ERN/LRPT reduced such postprandial hypertriglyceridemic response by almost 50%, showing an additional beneficial effect of niacin over statin treatment. This observation is in good accordance with a previous study showing that ERN lowered postprandial triglyceridemia in normolipidemic healthy volunteers.

By limiting postprandial hypertriglyceridemia, ERN/LRPT therapy reduces the atherogenicity of postprandial triglyceride-rich particles and their remnants. Indeed, during exaggerated postprandial response, the vessel wall is more exposed to proatherogenic remnants which can penetrate into the subintimal space by transcytosis, in a receptor-independent mechanism. TRL remnants are generated mainly by the action of LPL on CM and VLDL particles. In close relationship with the impact of ERN/LRPT on postprandial plasma levels of both apoCII and apoCIII, it is likely that ERN/LRPT, thus, accelerates hydrolysis of TG-rich lipoproteins, mainly chylomicrons and large VLDL-1 particles. Indeed, we presently observed that ERN/LRPT therapy was associated with an increase in plasma levels of apoCII, whereas those of apoCIII were slightly reduced postprandially; such postprandial variations in apoCII and apoCIII levels being not observed before ERN/LRPT treatment. ApoCII represents the most potent LPL activator, whereas apoCIII inhibits LPL activity; thus, the concomitant and opposite variations observed in apoCII and apoCIII after ER/LRPT therapy might favor intravascular catabolism of postprandial TRL particles. In addition, ERN/LRPT treatment abolished postprandial elevation in circulating levels of apoE, thus indicating a decrease in the generation of remnants particles. Taken together, those effects suggest that ERN/LRPT limits intravascular accumulation of TRL and their remnants and their further potential infiltration within the arterial wall.

We equally observed that ERN/LRPT abolished the postprandial elevation in endogenous CETP activity frequently observed in hyperlipidemic patients. Accelerated postprandial CETP-mediated CE transfer from HDL to TRL particles has been shown to favor the formation of CE-enriched remnants. An enhanced CETP activity induces the concomitant formation of TG-enriched/CE-poor HDL particles which represent good substrate for HL. Such particles are thus rapidly transformed into smaller HDL particles, contributing to the reduction of circulating HDLC levels and the establishment of a low HDLC phenotype. We thus propose that the ERN/
LPRT-induced limitation of postprandial CETP-mediated CE transfer from HDL to TRL particles represents one mechanism by which niacin exerts its anti-atherogenic action, specifically by correcting the lipoprotein distribution toward an anti-atherogenic lipoprotein profile characterized by reduced plasma levels of TRL remnants and increased plasma levels of large HDL2 particles. This is in good agreement with in vivo studies showing that the effect of niacin on plasma HDLC levels is intimately linked to CETP activity. In addition, the lipoprotein distribution observed after ERN/LPRT treatment display a high degree of homology with those observed after therapy with CETP inhibitors, suggesting a CETP inhibition-like effect of niacin.

We presently demonstrated that lipoprotein changes induced by ERN/LRPT treatment impact postprandial plasma cholesterol efflux capacity. Specifically, the capacity of postprandial plasma to stimulate cholesterol efflux via both ABCG1 and SR-BI was increased after ERN/LRPT treatment predominantly as a result of an increase in large HDL2 subspecies. These observations are in good agreement with previous studies showing an increase in plasma cholesterol efflux capacity from SR-BI and ABCG1 after niacin treatment in dyslipidemic patients. By contrast, we observed a decrease in the capacity of postprandial plasma to stimulate cholesterol efflux via ABCA1 after ERN/LRPT treatment. Pre-β-HDL particles represent the preferential acceptors of cholesterol via the ABCA1 pathway. These particles are generated in part by the dissociation of apoA1 from HDL particles after the concomitant action of CETP and HL. We presently observed that ERN/LRPT reduces the activity of CETP, thereby potentially limiting the generation of pre-β-HDL. This observation is consistent with a previous study showing a reduction, even nonsignificant, in the capacity of serum to stimulate cholesterol efflux via ABCA1 after niacin treatment. Finally, we observed that ERN/LRPT therapy slightly reduce postprandial plasma efflux capacity from human macrophages, indicating that the increase in cholesterol efflux via SR-BI and ABCG1 pathways failed to counterbalance the reduction in the ABCA1-dependent efflux. Earlier studies have demonstrated that the relative contribution of ABCA1 to human macrophage cholesterol efflux is predominant when compared with other specific pathways, mostly SR-BI, because ABCG1 has been shown to not contribute significantly to cholesterol efflux from human macrophage.
earlier studies, we presently observed no impact of niacin treatment on a background of statin therapy on cholesterol efflux to apoB-depleted serum despite elevation in circulating HDLC levels. It is relevant to consider that in this latter study, efflux was evaluated using J774 mouse macrophage cells, thus reflecting an ABCA1-dependent efflux mechanism. Taken together, these observations highlight the contribution of apoB-containing lipoproteins in the SR-BI-dependent cholesterol efflux from human macrophages.

Using hApoB/hCETP transgenic mice as a humanized mouse model, we evaluated the turnover of postprandial HDL particles isolated from niacin-treated patients. Interestingly, we demonstrated that in patients receiving ERN/LRPT treatment, HDL particles generated during postprandial phase display an improved capacity to mediate selective hepatic uptake of CE to the liver. Even though nonsignificant, we presently observed a minor reduction in apoCIII levels after ERN/LRPT treatment. This variation in apoCIII levels occurred concomitantly with an increase in plasma apoCII levels. ApoCII and apoCIII represent activator and inhibitor of lipoprotein lipase, respectively, thus suggesting that intravascular catabolism of TRL particles is accelerated after ERN/LRPT treatment. After LDL-mediated TG hydrolysis, redundant lipids surface and apolipoproteins are then transferred from TRL particles to HDL, thus contributing to formation of larger HDL particles. Interestingly, these latter HDL particles have been previously shown to display a higher ability to interact with SR-BI for selective uptake of CE by the liver. It is interesting to note that fasting HDL particles isolated from ERN/LRPT-treated patients displayed a reduced HDL-CE liver delivery as compared with their equivalent counterparts isolated from patients before ERN/LRPT treatment; however, an equivalent increase in HDL-mediated RCT to feces was observed, thus suggesting that niacin might increase cholesterol elimination via a liver-independent mechanism, such as the transintestinal cholesterol excretion pathway. Human ApoB/CETP double transgenic mice fed with niacin showed significant reduction in P2Y13 gene expression in both the liver and intestine. The niacin-induced reduction in P2Y13 expression in the liver has been previously proposed as potential mechanism underlying HDLC raising effect of niacin. In addition, niacin induced an increase in the intestinal expression of ABCA1 gene, which might equally contribute to the elevation of circulating levels of HDLC observed after niacin therapy. In the enterocyte, ACAT2 catalyzes the esterification of free cholesterol, providing cholesterol oleate and palmitate assembly into the lipid core of CM and VLDL. Its inhibition by niacin represents one potential mechanism for the reduction of apoB-containing lipoproteins assembly, leading to reduction of intestinal TRL particles synthesis during postprandial phase.
Two clinical trials, AIM-HIGH and HPS2-THRIVE, recently failed to demonstrate the benefit of adding niacin on a background of statin therapy to reduce cardiovascular outcomes. However, both trials involved patients who reached their LDL-C targets under aggressive lipid treatment, lowering considerably the chances to reveal a beneficial effect of niacin. A secondary analysis of the AIM-HIGH trial, performed in a subset of dyslipidemic patients displaying high TG (≥200 mg/dL) and low HDLC levels (<32 mg/dL), demonstrated a significant reduction of cardiovascular risk in the ER niacin group (hazard ratio: 0.64; P = 0.032). In addition, nonfasting triglycerides are associated with incidence of cardiovascular events in particular in patients displaying a low HDLC phenotype and elevated TG levels. Thus, there may be a subgroup of patients who might still benefit from niacin treatment. Whether the subtle beneficial effects observed in our study translate into clinical benefit (reduction of events) and whether they are sufficient to overcome the high rate of side effects observed with niacin is unclear and should be tested in an appropriate outcome trial.

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Disclosures
None.

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Extended-Release Niacin/Laropiprant Improves Overall Efficacy of Postprandial Reverse Cholesterol Transport

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MATERIAL AND METHODS

Study design
Dyslipidemic patients displaying a low HDL-C phenotype (<40mg/dl for men and ≤50 mg/dl for women) and triglycerides >150 mg/dl were recruited in this study. Patients presenting with diabetes, or with severe renal insufficiency or active liver disease were excluded. Subjects (5 males and 5 postmenopausal females) with a mean age of 64 ± 9 years were non obese (mean BMI = 26± 4 kg/m²) and non-smoker. All patients were on antihypertensive treatment (ACE inhibitor and/or Ca²⁺-channel blocker) and none was on any anti-diabetic medication. All patients received a statin therapy during a 4-week diet/statin run-in phase; 6 patients were treated with simvastatin (20-80 mg/d); 2 patients received fluvastatin 40/80 mg/d; 1 patient received atorvastatin 80 mg/d; 1 patient rosuvastatin 20 mg/d. Then, in addition to statin therapy, patients received ERN/LRPT 1 g/20 mg once-daily for 4 weeks, and ERN/LRPT 2 g/40 mg once-daily for 8 additional weeks. Before and after ERN/LPRT therapy, a postprandial time course was performed following consumption of a test meal representing a total energy of 875 kcal and containing 87g of fat, 5g of protein, 19g of carbohydrates and including 152mg of cholesterol. Subjects were asked to abstain from alcohol, smoking and vigorous exercise for 24 hours before the day of the test. Blood samples were collected in EDTA-containing tubes (final concentration of EDTA, 1mg/ml) immediately before test meal consumption and 2h, 4h, 6h and 8h after meal intake, and plasma was separated immediately by low-speed centrifugation (3000 rpm) for 10 min at 4°C. The study protocol was approved by the Ethics Committee of the Medical faculty of the University Munich, where the patients were recruited and followed up. The study was conducted in accordance with the ethical principles set forth in the Declaration of Helsinki. Written informed consent was obtained from all patients.

Lipoprotein fractionation and Biochemical analysis
Chylomicrons (CM; Sf>400) were isolated by centrifugation at 20,000 rpm for 45 min at 15°C using a SW41 Ti rotor in a Beckman XL70 ultracentrifuge ¹. Subfractions of triglyceride-rich lipoproteins, i. e. VLDL1 (Sf 60-400), VLDL2 (Sf 20-60) and IDL (Sf 12-20) were isolated from chylomicron free-plasma by nonequilibrium density gradient ultracentrifugation as previously described ². LDL and HDL subfractions were isolated from chylomicron free-plasma by isopycnic density gradient ultracentrifugation at 40,000 rpm for 44 h at 15°C as previously described ³. The lipid content of plasma and isolated lipoprotein fractions, total protein and apolipoproteins was quantified with a calibrated AutoAnalyzer (Konelab 20). Total cholesterol and triglyceride levels were determined with reagent kits from Roche Diagnostics and ThermoElectron, respectively. Free cholesterol and phospholipid concentrations were measured using reagents from Diasys. Cholesteryl ester (CE) mass was calculated as (TC-FC) x 1.67 and thus represents the sum of the esterified cholesterol and fatty acid moieties. Bicinchoninic acid assay reagent (Pierce) was utilized for protein quantification. Lipoprotein mass was calculated as the sum of the mass of the individual lipid and protein components for each lipoprotein fraction. Plasma LDL-cholesterol was calculated using the Friedewald formula. HDL-cholesterol levels were determined after dextran sulfate-magnesium precipitation of apolipoprotein B-containing lipoproteins. ApoAI and apoB concentrations were determined using immunoturbidimetric assays (ThermoElectron reagents and calibrators). ApoAII, apoE, apoCII and apoCIII levels were determined using immunoturbidimetric assays (Diasys reagents and calibrators). Plasma CETP mass was determined using commercial ELISA kit from Alpco Diagnostics (Salem, NH, USA).

Endogenous plasma CETP activity
Endogenous CE transfer from HDL to apoB-containing lipoproteins was performed as previously described. Radiolabeled HDL were obtained from the d>1.063 g/ml plasma fraction by ultracentrifugation at 100,000 rpm for 3h30 at 15°C with a Beckman TL100 centrifuge. Then d>1.063 g/ml fraction was labelled with [³H]-cholesterol (4 µCi/ml) at 37°C overnight. Radiolabeled [³H]-HDL were then isolated from the d>1.063 g/ml plasma fraction by centrifugation at 100,000 rpm for 5h30 at 15°C after adjustment of the density at 1.21 g/ml by addition of dry solid KBr. CETP-mediated cholesteryl ester transfer was determined after incubation of whole plasma from individual subjects at 37°C or 0°C for 3h in the presence of radiolabeled HDL (25 µg HDL-CE) and iodoacetate (final concentration 1.5 mmol/l) for inhibition of LCAT. After incubation, apolipoprotein B-containing lipoproteins were precipitated using the dextran sulfate-magnesium procedure. The radioactive content of the supernatant was quantified by liquid scintillation spectrometry with a Trilux 1450 (Perkin Elmer). Endogenous plasma CETP activity (expressed as percentage) was calculated as the amount of the label recovered in the supernatant after incubation and divided by the label present in the supernatant before incubation. The CETP-dependent CE transfer was calculated from the difference between the radioactivity transferred at 37°C and 0°C.

**Cholesterol efflux measurements**

Efflux assays were performed as previously described using human THP-1 macrophages and several cellular models Fu5AH, CHO-K1, CHO-hABCG1, and CHO-hABCA1. 3H-cholesterol-labeled cells were incubated 4 hours at 37°C in the presence of 40-fold diluted total plasma or 40-fold diluted apoB-depleted plasma. Cholesterol efflux to isolated HDL particles was measured by incubating cells in the presence of fixed HDL concentrations: 15 µgPL/mL for cholesterol loaded THP-1 macrophages; 10 µgPL/mL for Fu5AH (SR-BI-dependent efflux); 5 µgPL/mL for CHO-K1 and CHO-hABCG1; 10µgapoAI/ml for CHO-hABCA1) cells. ABCG1-dependent efflux was calculated as the difference between efflux to CHO-hABCG1 and CHO-K1 cells. In CHO- hABCA1, the expression of ABCA1 was induced by tetracycline (1 µg/mL). The ABCA1-dependent efflux was calculated as the difference between efflux to activated CHO-hABCA1 and non-activated cells. Fractional cholesterol efflux, (expressed as a percentage), was calculated as the amount of the label recovered in the medium divided by the total label in each well (radioactivity in the medium + radioactivity in the cells) obtained after lipid extraction from cells in a mixture of 3:2 hexane-isopropanol (3:2 v/v). Fractional cholesterol efflux was calculated as the amount of the label recovered in the medium divided by the total label in each well. The background cholesterol efflux obtained in the absence of any acceptor was subtracted from the efflux obtained with samples. A standard plasma was tested in all experiments and was used to calculate relative efflux of each sample. All efflux determinations were performed in triplicate for each sample. For all efflux assays, intra and inter assay coefficients of variation were 2% and 2.3%, respectively. The capacity of individual HDL subfractions to mediate FC efflux is expressed as a percentage of cholesterol efflux per mole of acceptor particle as previously described.

**In vitro Selective hepatic uptake of HDL-CE**

*In vitro* selective HDL-CE liver uptake was performed by using HepG2 cells as previously described. HepG2 cells were maintained in DMEM supplemented with 10% foetal bovine serum, 1% L-glutamine and 0.75% penicillin/streptomycin. Confluent cells were incubated in the presence of 3H-CE labelled HDL (60µg protein/ml) at 37°C for 5 hours. At the end of incubation, the medium was removed and cells were washed 4 times with PBS and incubated in the presence of an excess of unlabelled HDL (100µg protein/ml) for 30 minutes. Cells were then washed 4 times with PBS and solubilised with 200µl of NaOH 0.2N for 15 minutes at room temperature with gentle mixing. The radioactive content of 100µl of each cell lysate was
measured by liquid scintillation counting and the protein content in each well was determined. Selective uptake was calculated from the known specific radioactivity of radiolabeled HDL-CE and is expressed in $\mu$gHDL-CE/$\mu$g cell protein.

In vivo metabolic studies

**Animals:** hApoB/hCETP (ApoB/+;CETP+) double transgenic mice used in the present study were generated by breeding human CETP transgenic mice (line 5203) with mice transgenic for the human apoB gene have been previously characterised and described. Mice were housed in a conventional animal facility in a temperature-controlled room under a 12-hour light-dark cycle, weaned at 21 days, and fed ad libitum a normal mouse chow diet (RM1; Dietex France). For the in vivo studies, nine-weeks old female hApoB/hCETP transgenic mice were fed ad libitum a normal mouse chow diet supplemented or not with 1% Niacin during an 8-week period. During experiments, mice were anaesthetized using isoflurane (2%) and were euthanized using cervical dislocation. All animal procedures were performed at the Central Animal Facility of the Medical Faculty of La Pitié-Salpetriere Hospital with approval from the Direction Departementale des Services Veterinaires, Paris, France in accordance with institutional guidelines on Animal Experimentation and with a French Ministry of Agriculture license. In addition, the animals were handled according to investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health or the Directive 2010/63/EU of the European Parliament.

In vivo studies of the selective hepatic uptake of HDL-CE to feces cholesterol transport were performed as previously described. Radiolabeled HDLs were prepared as described above from postprandial plasma (0h and 8h after meal intake) obtained from patients with metabolic syndrome before and after ERN/LRPT treatment. $^3$H-CE labelled HDL ($10^5$ cpm/animal) was administered intravenously by retro-orbital injection. Blood samples (100µl) were drawn by retro-orbital puncture into heparinised capillary tubes at 3min, 2h, and 6h after injection. Feces were collected continuously over a 24 hour-period. At 24h after injection, mice were anesthetized using isoflurane (2%), exsanguinated, euthanized using cervical dislocation and perfused with saline buffer through the left ventricle. Plasma samples were used for liquid scintillation counting. The liver and intestine were collected, weighed, flash frozen and stored at -80°C. Liver (approximately 100 mg) were minced, transferred into counting vials and liquid scintillation solution was added for radioactivity measurement. Total feces were dried at 50°C for 5h, weighed and rehydrated overnight with water (57mg/ml). Feces were then homogenised and 200µl of samples were combined with 10 ml of liquid scintillation for counting. All counts were expressed as a percentage of the total injected $^3$H tracer dose.

RNA extraction, reverse-transcription and quantitative-PCR.

Total RNA from cell culture or tissues were extracted using the NucleoSpin RNA II kit (Macherey-Nagel) or TRIzol reagents (Euromedex), respectively, according to the manufacturer's instructions. Reverse transcription and real-time qPCR using a LightCycler LC480 (Roche) were performed as previously described. Expression of mRNA levels was normalized to mouse hypoxanthine phosphoribosyltransferase 1 (Hprt1), mouse non-POU domain containing, octamer-binding housekeeping gene (Nono), mouse heat shock protein 90kDa alpha (cytosolic), class B member 1 (Hsp90ab1). Data were expressed as a fold change in mRNA expression relative to control values.

Statistical analysis

The impact of ERN/LRPT treatment on all measured parameters was analysed using two-way repeated-measures ANOVA. Postprandial lipemia was quantified by calculating the area under the curve (AUC) and the incremental AUC (iAUC) for all measures. The iAUC
represents the increase in area in response to the test meal relative to before meal intake. Results were considered statistically significant at p<0.05.

References


Supplemental Figure I. Line plots showing plasma concentrations of LDL-C and apoB levels (panel A), and HDL-C, ApoAI and ApoAII (Panel B) determined throughout the postprandial state in patients (n=10) before (closed circles) and after 12 weeks of ERN/LRPT treatment (open circles). Histograms showing values of AUC, before (closed bars) and after (open bars) ERN/LRPT treatment of LDL-C and HDL-C, HDL2-C, HDL3-C. Values are mean ± SEM. *P<0.001 versus before ERN/LRPT treatment.
Supplemental Figure II. Line plots showing the capacity of postprandial apoB-depleted plasma to mediate cellular free cholesterol efflux from THP-1 (Panel A), and via ABCA1 (panel B), SR-BI (Panel C) and ABCG1 (Panel D). Cholesterol efflux from cells was determined after 4h incubation in the presence of 40-fold diluted apoB-depleted plasma from patients before (closed circles) and after ERN/LRPT treatment (open circles). Graphs showing AUC for cellular cholesterol efflux before (closed bars) and after ERN/LRPT therapy (open bars) are presented. Values are mean ± SEM. *P<0.05 versus before ERN/LRPT treatment.
Supplemental Figure III. Line plots showing the capacity of postprandial HDL subfractions isolated from patients (n=10) before (solid line) and after 12 weeks of ERN/LRPT treatment (dotted line) to mediate free cholesterol efflux from THP-1 (Panel A), and via ABCA1 (Panel B), SR-BI (Panel C) and ABCG1 (Panel D). Cholesterol efflux from cells was determined after 4h incubation in the presence of isolated HDL subfractions and is expressed per mole of HDL particles. Cholesterol efflux to isolated HDL particles was measured by incubating cells in the presence of fixed HDL concentrations: 15 µgPL/mL for cholesterol loaded THP-1 macrophages; 10 µgPL/mL for Fu5AH (SR-BI–dependent efflux); 5 µgPL/mL for CHO-K1 and CHO-hABCG1; 10µgapoAl/ml for CHO-hABCA1 cells.