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Free cholesterol transfer to high-density lipoprotein (HDL) upon triglyceride lipolysis underlies the U-shape relationship between HDL-cholesterol and cardiovascular disease

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Abstract

Background: Low concentrations of high-density lipoprotein cholesterol (HDL-C) represent a well-established cardiovascular risk factor. Paradoxically, extremely high HDL-C levels are equally associated with elevated cardiovascular risk, resulting in the U-shape relationship of HDL-C with cardiovascular disease. Mechanisms underlying this association are presently unknown. We hypothesised that the capacity of high-density lipoprotein (HDL) to acquire free cholesterol upon triglyceride-rich lipoprotein (TGRL) lipolysis by lipoprotein lipase underlies the non-linear relationship between HDL-C and cardiovascular risk.

Methods: To assess our hypothesis, we developed a novel assay to evaluate the capacity of HDL to acquire free cholesterol (as fluorescent TopFluor® cholesterol) from TGRL upon in vitro lipolysis by lipoprotein lipase.

Results: When the assay was applied to several populations markedly differing in plasma HDL-C levels, transfer of free cholesterol was significantly decreased in low HDL-C patients with acute myocardial infarction (−45%) and type 2 diabetes (–25%), and in subjects with extremely high HDL-C of >2.59 mmol/L (>100 mg/dL) (−20%) versus healthy normolipidaemic controls. When these data were combined and plotted against HDL-C concentrations, an inverse U-shape relationship was observed. Consistent with these findings, animal studies revealed that the capacity of HDL to acquire cholesterol upon lipolysis was reduced in low HDL-C apolipoprotein A-I knock-out mice and was negatively correlated with aortic accumulation of [^{3}H]-cholesterol after oral gavage, attesting this functional characteristic as a negative metric of postprandial atherosclerosis.

Conclusions: Free cholesterol transfer to HDL upon TGRL lipolysis may underlie the U-shape relationship between HDL-C and cardiovascular disease, linking HDL-C to triglyceride metabolism and atherosclerosis.

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Introduction

Low levels of plasma high-density lipoprotein cholesterol (HDL-C) represent a well-established cardiovascular risk factor.[1,2] However, therapeutic raising of HDL-C on a background of statin treatment does not exert expected beneficial effects on cardiovascular disease (CVD).[3] Furthermore, large-scale epidemiological studies reveal that both cardiovascular and overall mortality are paradoxically increased at extremely high HDL-C levels,[4–6] an observation which might account for the negative results of HDL-C-raising trials. It has therefore been suggested that it is not HDL-C itself that is causatively related to atheroprotection but, rather, a cardioprotective HDL function, which cannot always be reliably estimated through the assay of HDL-C.[7]

The major atheroprotective function of HDL is presently thought to involve cholesterol efflux from arterial wall cells with its subsequent transport to the liver for excretion in a process of reverse cholesterol transport (RCT).[7] Such a 'HDL flux hypothesis' is based on negative associations of CVD with in vitro measurements of cellular cholesterol efflux from lipid-loaded macrophages.[8] Cholesterol efflux from tissue macrophages, however, provides only a small contribution to HDL-C levels if any[9] and can therefore hardly explain the complex non-linear association between HDL-C and cardiovascular risk.

Intravascular high-density lipoprotein (HDL) metabolism is intimately linked to that of triglyceride-rich lipoproteins (TGRLs), a phenomenon which is frequently manifested as a negative correlation between plasma levels of HDL-C and triglycerides.[10,11] Given that elevated triglyceride levels are increasingly accepted as a cardiovascular risk factor,[10,11] low HDL-C has been proposed to represent a biomarker of elevated concentrations of atherogenic TGRL remnants,[10] a clinically attractive concept which, however, lacks mechanistic insight. In the circulation, HDL and TGRL interact via multiple metabolic pathways, the most studied of which includes heteroexchange of core lipids mediated by cholesteryl ester transfer protein (CETP).[12] A key pathway linking HDL and TGRL involves transfer to HDL of surface remnants generated during TGRL lipolysis by lipoprotein lipase (LPL).[13] This frequently overlooked process delivers high amounts of free cholesterol to HDL and constitutes a quantitatively major source of circulating HDL-C, accounting for up to 50% of its variation.[14] Indeed, intestinal cholesterol secretion within chylomicrons is comparable with net cholesterol efflux from all extra-hepatic tissues combined.[15,16] Mechanistically, free cholesterol acquirement by HDL can be primarily mediated by small-sized complexes containing phospholipid and apolipoprotein (apo)A-I, the major HDL protein and potent biological detergent.[17,18] Remarkably, high amounts of apoA-I are synthesised by the intestine to be incorporated into chylomicrons and postprandially transferred to HDL.[19]

To explain the relationship between HDL-C levels and cardiovascular risk, we propose a reverse remnant cholesterol transport (RRT) hypothesis which involves acquirement by HDL of surface remnants of TGRL upon lipolysis with subsequent transport of remnant-derived cholesterol to the liver in a pathway which originates in the intestine with the secretion of apoA-I on chylomicrons followed by their transport to plasma via lymph. As a corollary, we suggest that plasma concentration of HDL-C is an imperfect static measure of cholesterol flux through this dynamic pathway.

To assess this hypothesis, we developed an in vitro fluorescent assay to evaluate the capacity of HDL to acquire free cholesterol from TGRL during lipolysis by LPL. The assay was applied to several groups of subjects markedly differing in HDL-C levels. Our findings reveal decreased free cholesterol transfer from TGRL to HDL in subjects with both low and extremely high HDL-C, a finding which may link HDL-associated cardiovascular risk to triglyceride metabolism and account for the U-shape relationship of HDL-C and CVD.

Methods

Study populations

Four groups of subjects markedly differing in HDL-C levels were studied together with corresponding control groups. Male patients presenting with ST segment elevation acute myocardial infarction (AMI; $n = 22$) were recruited at the Heart Institute-InCor University of Sao Paulo Medical School Hospital (São Paulo, Brazil) before initiation of any treatment within no later than 24 h of clinical presentation in the Emergency Room. Healthy non-smoking normolipidaemic male subjects ($n = 8$) were recruited as controls. Treatment-naïve patients with well-controlled type 2 diabetes (T2D; males and postmenopausal women, $n = 17$) were

recruited from the Ramon Carrillo Centre (La Matanza, Buenos Aires, Argentina). Healthy non-smoking normolipidaemic subjects ($n = 16$) were recruited from the same geographical area to constitute the control group. Subjects with high (1.81 to 2.59 mmol/L (70 to 100 mg/dL); $n = 20$) and extremely high (>2.59 mmol/L (>100 mg/dL); $n = 20$) HDL-C levels together with healthy normolipidaemic control volunteers ($n = 20$) were recruited at the La Pitié-Salpetrière University Hospital (Paris, France).

Measurement of HDL capacity to acquire free cholesterol during TGRL lipolysis by LPL

Given that fluorometric measurements are distinguished by high sensitivity and specificity, introduction in the assay of a fluorescent free cholesterol derivative provides an excellent means for evaluating free cholesterol transfer across lipoproteins. Importantly, selective labelling of TGRL with fluorescent free cholesterol implies its absence from HDL at baseline, thereby strongly enhancing its relative enrichment in HDL over time as compared with that of unlabelled free cholesterol. We therefore labelled TGRL with fluorescent 23-(dipyrrometheneboron difluoride)-24-norcholesterol (Avanti Polar Lipids, Alabaster, AL, USA), also known as TopFluor® cholesterol (TopF) and BODIPY-cholesterol, and evaluated its transfer to HDL during LPL-induced lipolysis.

To isolate TGRL (d < 1.019 g/ml), a single-step ultracentrifugation was used. HDL was employed in the form of ultracentrifugally isolated HDL (UC-HDL) or as apoB-depleted plasma. To label human TGRL with TopF, TGRL was added to lipoprotein-deficient plasma (LPDP) at the LPDP:TGRL ratio of 1:100 by volume. The mixture was filtered and TopF was added as a chloroformic solution followed by overnight incubation at 37°C under gentle stirring. Labelled TGRL was separated from unbound fluorescent lipid using filtration through a PD-10 Sephadex column. Triglyceride concentration in purified labelled TGRL was measured by photometry and TopF fluorescence was registered at the excitation/emission wavelengths of 500/525 nm using a Gemini Microplate Reader (Molecular Devices, USA) to verify the labelling.

To evaluate the transfer of TopF, Tris buffer (0.4 M, pH 8) was mixed on ice with required amounts of TopF-labelled TGRL and HDL, and LPL from *Pseudomonas* sp. or from bovine milk (both from Sigma, France) was added to start lipolysis at 37°C. At the end of the incubation, the reaction mixture was placed on ice and apoB precipitant containing phosphotungstic acid and $MgCl_2$ was added. Following incubation at room temperature and centrifugation, HDL-containing supernatant was aspirated, filtered and transferred to a black microplate for fluorescence reading. Fluorescence of the standard TGRL sample alone was also measured at a triglyceride concentration employed in the assay and fluorescent values measured in HDL were expressed as a percentage of fluorescence of such standard sample. In some experiments, inhibitors for CETP (torcetrapib, 25 μM) and lecithin-cholesterol acyltransferase (LCAT) (iodoacetamide, 750 μM) were used.

To characterise the transfer of TopF to HDL obtained from clinical plasma samples, TGRL and apoB-depleted plasma were used at final concentrations of 0.34 mmol/L (30 mg triglyceride/dL) and 3.3%, respectively. Assay conditions (lipoprotein concentrations, incubation time, etc.) were chosen to model those of postprandial TGRL lipolysis in humans. A reference apoB-depleted plasma sample obtained from one healthy normolipidaemic control plasma aliquoted and stored at −80°C was included in each series of measurements and all values obtained in clinical samples were normalised to that observed in the reference plasma.

Postprandial aortic cholesterol accumulation in mice

Postprandial aortic accumulation of free cholesterol was evaluated in vivo after gavage with 100 μCi of [^{3}H]-cholesterol administrated with olive oil to three groups of mice markedly differing in HDL-C levels, including low-HDL-C apoA-I knock-out mice, high-HDL-C human apoA-I transgenic mice and control wild-type mice. The animals were euthanised 2 h after the gavage, their aortas removed and weighed, and specific radioactivity measured. HDL capacity to acquire TopF from TGRL during LPL-induced lipolysis was evaluated in apoB-depleted EDTA plasma obtained from every mouse following overnight fast several weeks before the gavage experiment.

A detailed description of the methods is provided in the Supplementary Material online.

Results

HDL isolated by ultracentrifugation or apoB depletion readily acquired TopF upon lipolysis of TopF-labelled TGRL (Figure 1; Supplementary Figure 1). The transfer was time-dependent and typically saturated after 1–2 h, except for the highest concentration of UC-HDL (Figure 1(a) and (c)). Dose-dependence of the transfer was characterised by an increase with increasing UC-HDL concentrations (Figure 1(b)), while maximal TopF accumulation in HDL isolated by apoB depletion was observed at intermediate concentrations of apoB-depleted plasma, decreasing at higher concentrations (Figure 1(d)). This dose-dependence

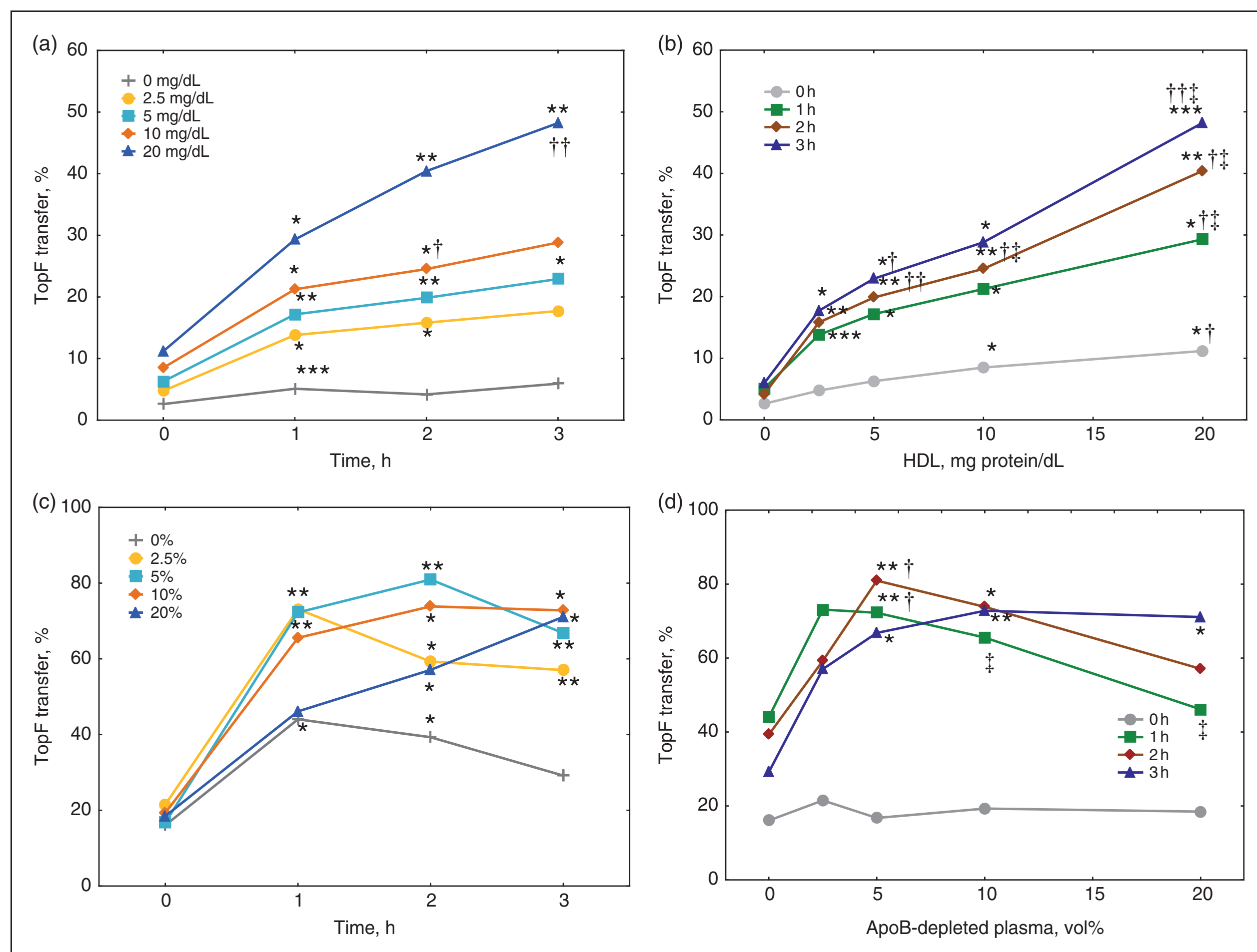

Figure 1. Kinetics and dose-dependences of the transfer of fluorescent free cholesterol from triglyceride-rich lipoprotein (TGRL) to high-density lipoprotein (HDL) during lipoprotein lipase (LPL)-induced lipolysis. HDL was isolated from normolipidaemic EDTA plasma by density gradient ultracentrifugation ((a) and (b)) or by apolipoprotein B (apoB) depletion ((c) and (d)) and incubated for up to 3 h at 37°C with LPL (190 U/mL) and TGRL (30 mg triglyceride/dL) labelled with TopFluor® cholesterol (TopF), at a final concentration of 2.5–20 mg HDL protein/dL ((a) and (b)) or at a final dilution of apoB-depleted plasma of 2.5 to 20% v/v ((c) and (d)), respectively. At the end of the incubation, HDL was separated from TGRL by apoB depletion and TopF fluorescence was measured in HDL at the excitation/emission wavelengths of 500/525 nm. Mean values of 4–6 independent experiments performed in duplicate with four individual HDL samples are shown. Please note that means with SDs are shown in Supplementary Figure 1 for the sake of clarity.
$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* 0 h; $^{\dagger\dagger}p < 0.01$, $^{\dagger}p < 0.05$ *vs.* 1 h ((a) and (c)).
$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* 0 mg/dL; $^{\dagger\dagger}p < 0.01$, $^{\dagger}p < 0.05$ *vs.* 2.5 mg/dL; $^{\ddagger}p < 0.05$ *vs.* 5 mg/dL (b)
$^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* 0%; $^{\dagger}p < 0.05$ *vs.* 2.5%; $^{\ddagger}p < 0.05$ *vs.* 5% (d).

was confirmed by measurements of unlabelled native free cholesterol (Supplementary Figure 2), revealing a correlation coefficient of 0.71 ($p < 0.001$) between the two methods and demonstrating the validity of our fluorescence-based approach. In addition, when HDL was re-isolated from the same reaction mixtures by apoB precipitation and ultracentrifugation, TopF fluorescence readings in such HDL were strongly correlated ($r = 0.95$, $p = 0.014$), further validating our approach.

When V_{max} and K_d for the transfer of unlabelled free cholesterol were calculated, they revealed maxima at intermediate concentrations of HDL (Figure 2(a)), consistent with the results obtained using fluorescent free cholesterol. As a consequence, concentration of apoB-depleted plasma of 3.3% and UC-HDL concentration of 4 mg protein/dL, both corresponding to the initial concentration-dependent sections of the dose-dependent curves (Figure 1(b) and (d)), were chosen to compare individual plasma samples for the capacity of their HDL to acquire free cholesterol.

Initial characterisation of the assay revealed good reproducibility of the measurement of TopF transfer to normolipidaemic apoB-depleted plasma, with intra- and inter-assay coefficients of variation of 5.1% and 8.8%, respectively ($n = 10$). The assay was initially

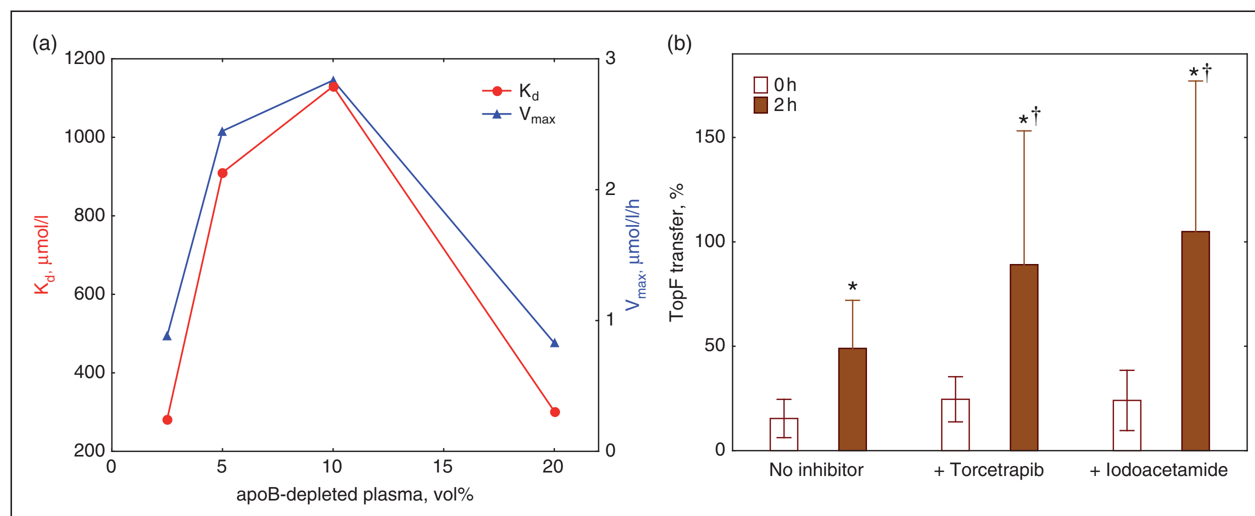

Figure 2. Kinetic parameters of free cholesterol transfer from triglyceride-rich lipoprotein (TGRL) to high-density lipoprotein (HDL) during lipoprotein lipase (LPL)-induced lipolysis and effects of inhibitors. HDL was isolated from normolipidaemic EDTA plasma by apolipoprotein B (apoB) depletion and incubated for 2 h at 37°C with lipoprotein lipase (LPL) (190 U/mL) and TGRL (7.5, 15, 30 and 60 mg TG/dL) at a final dilution of apoB-depleted plasma of 2.5, 5, 10 and 20% v/v. At the end of the incubation, HDL was separated from TGRL by apoB depletion, free cholesterol was measured in HDL using the Amplex Red kit and K_d and V_{max}. were calculated according to Lineweaver–Burk. To evaluate the effects of inhibitors, ultracentrifugally isolated HDL (4 mg protein/dL) isolated from normolipidaemic EDTA plasma was incubated for 2 h with LPL (190 U/ml) and TopFluor® cholesterol-labelled TGRL (30 mg triglyceride/dL) in the absence or presence of torcetrapib (25 µM) or iodoacetamide (750 µM). Data from three independent experiments performed in duplicate with three individual HDL samples are shown.
$^*p < 0.05$ *vs.* 0 h; $^{\dagger}p < 0.05$ *vs.* 2 h incubation in the absence of the inhibitors.

employed to obtain insight into mechanisms of free cholesterol transfer from TGRL to HDL during LPL-mediated lipolysis using inhibitors. Both inhibition of CETP using torcetrapib and inhibition of LCAT using iodoacetamide markedly (1.8- and 2.1-fold, respectively) enhanced the accumulation of TopF in UC-HDL (Figure 2(b)). Torcetrapib equally increased the accumulation of TopF when apoB-depleted plasma was used as a source of HDL (from 49 ± 11% to 74 ± 11 in the absence and presence of 25 µM torcetrapib, respectively, in 10% apoB-depleted plasma; $n = 4$, $p < 0.05$), modifying the dose-dependence of the transfer in such a way that its decrease at high concentrations of apoB-depleted plasma was abolished.

The assay was subsequently applied to several groups of patients markedly differing in plasma HDL-C. Patients with AMI featured reduced HDL-C (−31%, $p < 0.001$; Supplementary Table 1) relative to healthy normolipidaemic controls. The capacity of HDL to acquire TopF was decreased in AMI patients by −46% (controls, 103 ± 12% *vs.* patients, 57 ± 28%, $p < 0.001$; Figure 3(a)). This decrease was thereby more pronounced as compared with that in HDL-C levels. The capacity of HDL to acquire TopF was positively correlated with HDL-C in this low HDL-C group ($r = 0.49$, $p < 0.05$). By contrast, accumulation of TopF in UC-HDL isolated from AMI plasma was similar to that observed in UC-HDL from controls when the lipoproteins were compared on a protein concentration basis (Supplementary Figure 3(a)). To evaluate whether between-group differences were specifically related to the transfer of fluorescent cholesterol, we measured the accumulation of unlabelled native free cholesterol in HDL from a subpopulation of AMI and control subjects. The transfer of free cholesterol was strongly (6.5-fold, $p < 0.01$) reduced in AMI relative to control HDL (Supplementary Figure 3(b)) and was correlated with measurements of TopF-cholesterol ($r = 0.73$, $p = 0.005$).

Treatment-naïve patients with well-equilibrated T2D, an established cardiovascular risk factor, were characterised by a typical lipid profile involving reduced HDL-C (−21%, $p < 0.05$) and elevated triglycerides (+94%, $p < 0.05$) as compared with healthy normolipidaemic controls (Supplementary Table 2). The capacity of HDL to acquire free cholesterol was decreased in T2D patients by −24% (controls, 96 ± 28% *vs.* patients, 72 ± 33%, $p < 0.05$; Figure 3(a)). This decrease was similar to that in HDL-C levels.

As compared with healthy, normolipidaemic controls, subjects with high HDL-C levels (from 1.81 to 2.59 mmol/L (70 to 100 mg/dL)) were characterised by elevated HDL-C (+34%, $p < 0.001$; Supplementary Table 3). Despite such marked increase, the capacity of HDL to acquire free cholesterol did not differ between the groups (controls, 97 ± 22% *vs.* patients,

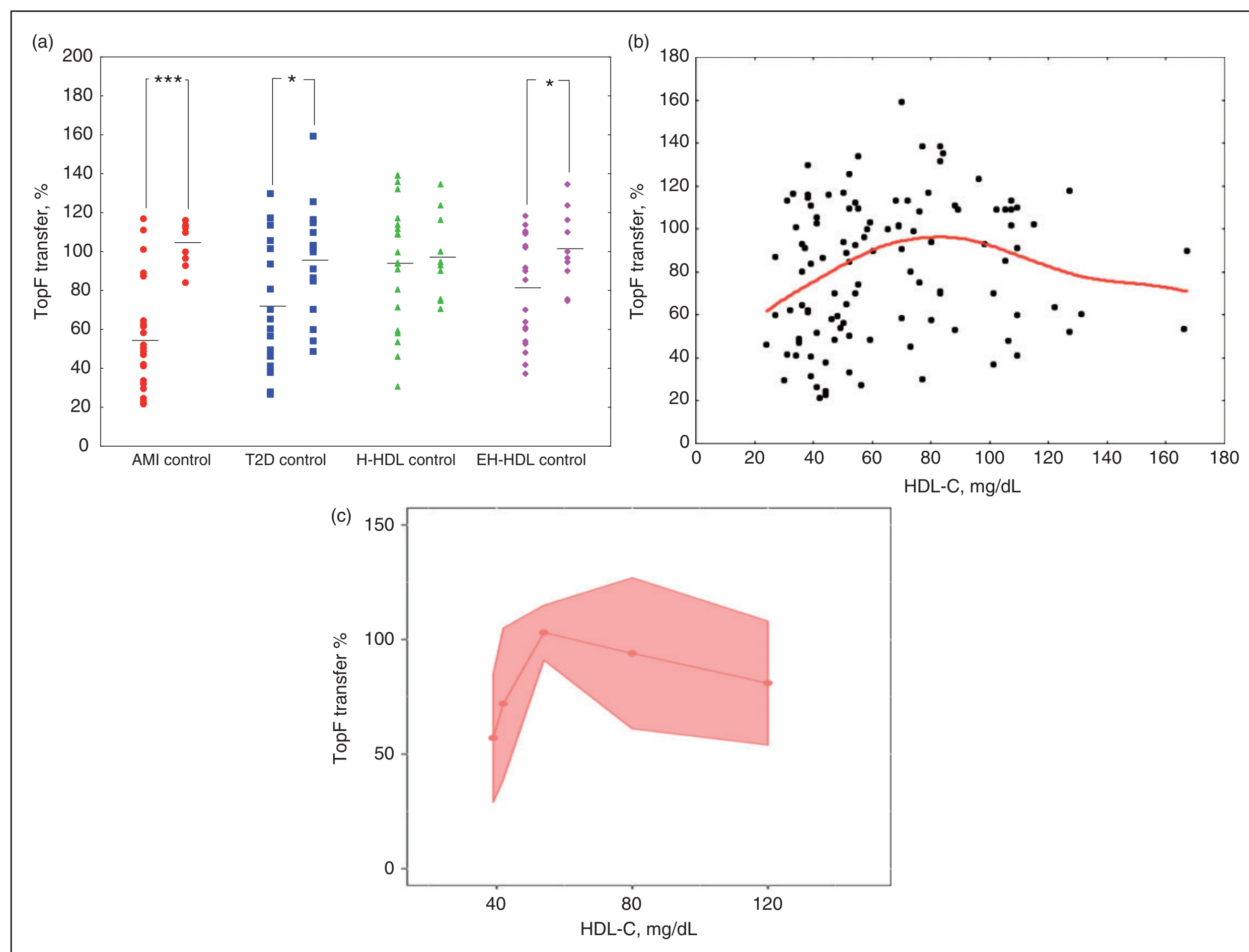

Figure 3. Transfer of fluorescent free cholesterol from triglyceride-rich lipoprotein (TGRL) during lipoprotein lipase (LPL)-induced lipolysis to high-density lipoprotein (HDL) isolated from patients with acute myocardial infarction (AMI; $n = 22$), type 2 diabetes (T2D; $n = 17$), high HDL cholesterol (H-HDL; $n = 20$) and extremely high HDL-C (EH-HDL; $n = 20$). HDL was isolated from EDTA plasma by apolipoprotein B (apoB) depletion and incubated for 2 h at 37°C with LPL (190 U/mL) and TopFluor® cholesterol (TopF)-labelled TGRL (30 mg triglyceride/dL) at a final concentration of apoB-depleted plasma of 3.3% v/v. At the end of the incubation, HDL was separated from TGRL by apoB depletion and TopF fluorescence was measured at the excitation/emission wavelengths of 500/525 nm. The horizontal lines depict mean values in each group; ***$p < 0.001$, *$p < 0.05$ *vs*. HDL from corresponding healthy normolipidaemic controls (a). The relationship between TopF transfer and plasma HDL-C levels was plotted using least-square data fitting for all individual subjects studied (b) as well as for the means ± SDs of TopF transfer and HDL-C in each group using ggplot2 (c). In (c), all controls were combined to form a single control group.

94 ± 33%, Figure 3(a)), thereby diverging from the HDL-C assay.

In subjects with extremely high HDL-C concentrations (>2.59 mmol/L (>100 mg/dL)), HDL-C was elevated almost 2-fold (+92%) relative to controls (Supplementary Table 4). Despite such pronounced increase, the capacity of HDL to acquire free cholesterol was diminished by −20% in this group (controls, 101 ± 20% *vs*. patients, 81 ± 27%, $p < 0.05$; Figure 3(a)), further diverging from HDL-C. As a result, no correlation between HDL capacity to acquire TopF and plasma HDL-C was observed across all the populations studied ($r = 0.10$, $p = 0.29$; $n = 123$). Such absence of linear correlation was related to a complex non-linear dependence between TopF transfer and HDL-C observed across both individual subjects (Figure 3(b)) and studied populations (Figure 3(c)), which resembled an inverse U-shape. It is of note that no difference in the capacity of HDL to acquire TopF was observed between the four control groups, validating our study design (Figure 3(a)).

To evaluate potential determinants of the reduced free cholesterol transfer to HDL, plasma activities of CETP and LCAT were assessed in the patients using fluorometric kits. The only significant difference observed relative to controls involved reduced CETP activity in AMI patients (−50%, $p < 0.01$; Supplementary Figure 4).

Given that subjects with low and high HDL-C levels greatly differ in their HDL particle profile, with large HDL prevailing at high HDL-C,[20] we evaluated to whether HDL particle subpopulations differed in their capacity to acquire free cholesterol upon TGRL lipolysis by LPL. We found that large, light HDL2 particles were up to 3.8-fold less efficient acceptors for TopF relative to small, dense HDL3 at a fixed protein concentration of 4 mg/dL (Supplementary Figure 5), equivalent to a 2.8-fold per particle difference.

The relationship of the assay with CVD was initially evaluated on the basis of published mortality data. When cardiovascular and overall mortality data obtained from the large-scale CANHEART HDL study[4] and Copenhagen City Heart Study[6] were recalculated for the mean HDL-C levels observed in the five populations studied by us (AMI, T2D, subjects with high and extremely high HDL-C, and pooled controls) and plotted against HDL-C, a U-shape relationship was observed as reported[4,6] (Supplementary Figure 6(a)). In striking contrast, when HDL-C was replaced by the mean values of HDL capacity to acquire TopF, linear negative relationships with both cardiovascular and overall mortality were obtained (r^2 from 0.89 to 0.98, p from 0.044 to 0.003), indicative of the significant relationship of mortality with the assay outcome (Supplementary Figure 6(b)).

Pathophysiological relevance of the assay was further evaluated in vivo using murine models displaying marked differences in circulating HDL-C levels, specifically wild-type mice, dyslipidaemic low HDL-C apoA-I –/– mice and high HDL-C mice transgenic for human apoA-I. As expected, HDL-C concentrations were reduced by −82% in apoA-I knock-out mice and elevated by +72% in human apoA-I transgenic animals (Figure 4(a)). Expectedly, CETP activity was nearly absent from plasma of all mice and LCAT activity was greatly reduced relative to human plasma (Supplementary Figure 7), with apoA-I knock-out animals featuring the lowest LCAT activity.

The capacity of HDL to acquire free cholesterol measured upon TGRL lipolysis by LPL in vitro using TopF was reduced by −38% ($p<0.01$) in apoA-I knock-out mice but was not modified in human apoA-I transgenic animals (Figure 4(b)). When all the mice received an oral gavage of [^{3}H]-cholesterol together with olive oil, postprandial accumulation of [^{3}H]-cholesterol in the aorta was significantly (6.1-fold) enhanced 2 h after the gavage in apoA-I knock-out relative to wild-type mice, while no difference between wild-type and human apoA-I transgenic mice was found (Figure 4(c)). As a consequence, the capacity of HDL to acquire free cholesterol in vitro was significantly and negatively correlated with the aortic accumulation of [^{3}H]-cholesterol in vivo across all mice, attesting this functional parameter as a negative metric of postprandial atherosclerosis (Figure 4(d)).

Discussion

In the present study, we evaluated our RRT hypothesis that free cholesterol transfer to HDL upon TGRL lipolysis by LPL underlies the complex relationship between HDL-C levels and cardiovascular risk. We provide evidence, using human subjects and animal models markedly differing in HDL-C concentrations, that this process links triglyceride metabolism to atherosclerosis and may account for the relationship between HDL-C and CVD.

To assess our hypothesis, we developed an original method to evaluate in vitro free cholesterol transfer from TGRL to HDL upon LPL-mediated lipolysis of TGRL labelled with TopF, a fluorescent derivative of free cholesterol. Earlier studies of material exchange between TGRL and HDL revealed that multiple lipid and protein components, including free cholesterol, phospholipid and apolipoproteins, were transferred from TGRL to HDL upon LPL-mediated lipolysis.[13,14,21,22] Mechanisms of such exchange are defined by the removal of excess surface fragments from TGRL, which cannot adapt the structure of remnant particles possessing reduced hydrophobic core. Formation of surface TGRL remnants possibly involves spontaneous organisation of excessive surface lipids separated from TGRL into low-energy complexes which resemble micelles or liposomes.[23]

ApoA-I, the major HDL protein present in high amounts on chylomicrons,[19] can play a central role in this process as a result of its distinct lipid-binding properties.[17,18] Indeed, apoA-I avidly interacts with minimal amounts of lipids present in aqueous solutions, transforming them into highly organised lipoprotein complexes in the HDL density range.[17,18] While apoA-I can solubilise excess surface lipids released from TGRL upon lipolysis, HDL phospholipid may provide lipid surface required for their absorption.[21,23,24] Other structural HDL apolipoproteins, including apoA-II, apoE and apoM, can equally be expected to participate in lipid transfer during lipolysis, consistent with the presence of free cholesterol acceptor activity in HDL from apoA-I –/– mice.

As lipid transfer proteins play key roles in exchanging lipids across lipoproteins, these proteins may actively participate in the transfer of surface lipids upon LPL-mediated lipolysis. To assess this possibility, we employed torcetrapib, a specific CETP inhibitor, and observed enhanced transfer of free cholesterol upon inhibition. Such an inhibitory role of CETP towards cholesterol removal from TGRL can be

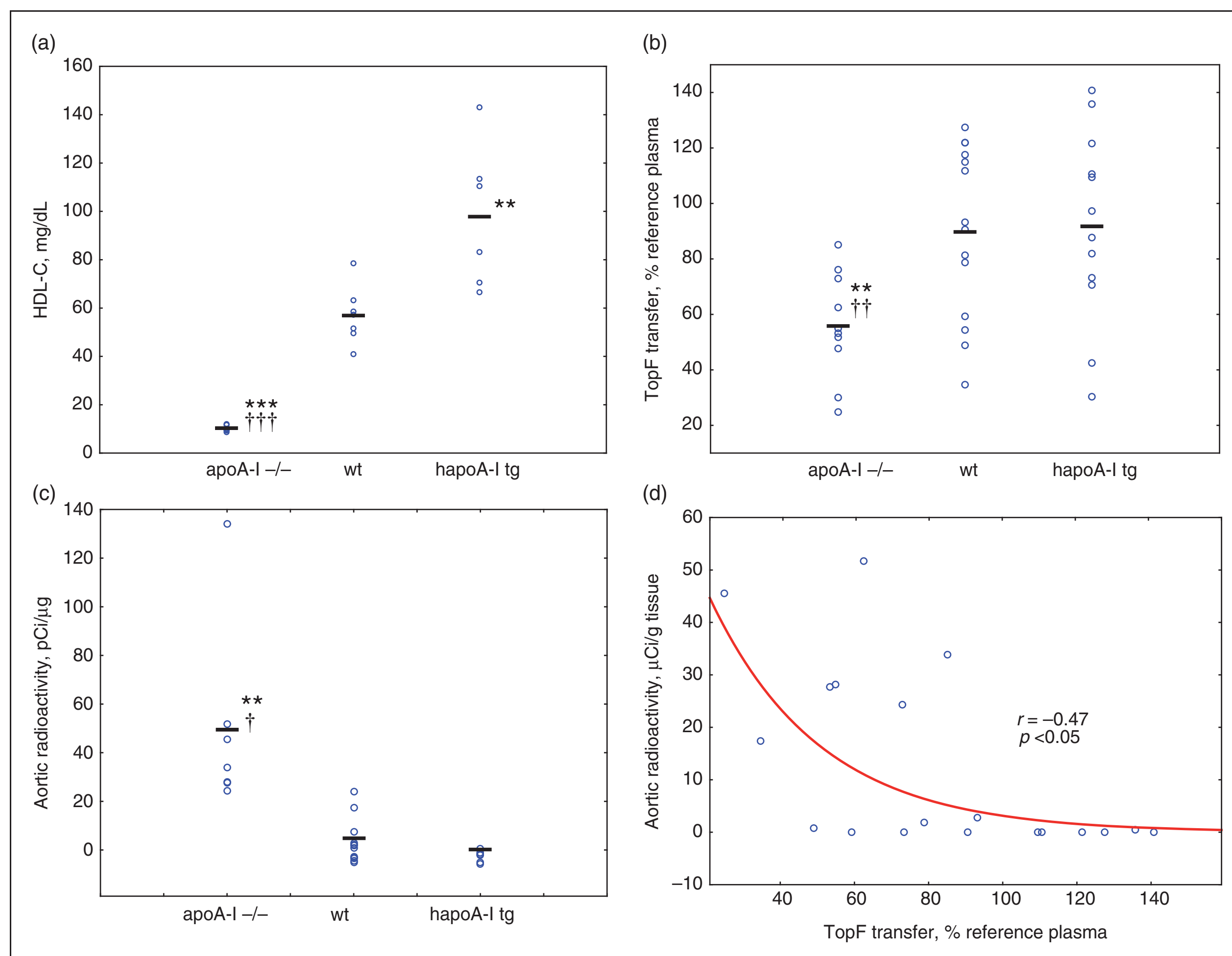

Figure 4. Relationships of plasma high-density lipoprotein cholesterol (HDL-C) levels and high-density lipoprotein (HDL) capacity to acquire fluorescent free cholesterol from triglyceride-rich lipoprotein (TGRL) upon lipoprotein lipase (LPL)-induced lipolysis with postprandial aortic cholesterol accumulation in mice. HDL-C concentrations (a) and capacity of HDL (as 3.3% apolipoprotein (apo)B-depleted EDTA plasma); (b) to acquire TopF from TGRL (30 mg triglyceride/dL) during LPL-induced lipolysis were measured in human apoA-I transgenic (hapoA-I tg; $n = 6–12$), apoA-I knock-out (apoA-I -/-; $n = 6–10$) and control wild-type (wt; $n = 7–14$) mice. Postprandial aortic accumulation of cholesterol (c) was evaluated in vivo after gavage with 100 µCi of [^{3}H]-cholesterol administered with olive oil (100 µL). The animals were euthanised 2 h after the gavage, their aortas removed and specific radioactivity measured per wet tissue weight. The relationship between postprandial aortic accumulation of [^{3}H]-cholesterol in vivo and capacity of HDL to acquire TopFluor® cholesterol (TopF) from TGRL during LPL-mediated lipolysis in vitro was plotted using exponential data fitting (d). The horizontal lines depict mean values in each group.
$^{***}p < 0.001$, $^{**}p < 0.01$ *vs.* wild-type mice.
$^{†††}p < 0.001$, $^{††}p < 0.05$, $^{†}p < 0.05$ *vs.* human apoA-I transgenic mice.

explained by its capacity to transfer cholesterol, in a form of cholesteryl ester, from HDL back to TGRL.[12] Indeed, HDL represents a major source of cholesteryl ester in human plasma, reflecting preferential association of LCAT with HDL.[16] When free cholesterol is transferred from TGRL to HDL and esterified under the action of LCAT, a molecule of cholesteryl ester formed becomes a substrate for CETP and can be readily transferred back to TGRL in exchange for a molecule of TGRL-derived triglyceride. This mechanism can be operative for TopF as it is efficiently esterified by LCAT and as its ester is transferred by CETP in vitro.[25,26]

If this mechanism is true and LCAT and CETP act along the same pathway of cholesterol removal from HDL, then inhibition of LCAT should enhance LPL-induced accumulation of TGRL-derived free cholesterol in HDL. Consistent with this conclusion, we did observe accelerated accumulation of TopF upon LCAT inhibition by iodoacetamide, suggesting a multi-step mechanism of free cholesterol movement between HDL and TGRL upon LPL-induced lipolysis, which

involves acquirement of free cholesterol by HDL with its subsequent esterification by LCAT followed by CETP-mediated exchange of the generated cholesteryl ester for triglyceride present in TGRL.

Importantly, kinetic data reveal that esterification of free cholesterol by LCAT occurs more slowly than both facilitated diffusion of cholesteryl ester mediated by CETP and free cholesterol transfer across lipoproteins,[16,27,28] thereby constituting a rate-limiting step in the exchange of cholesterol between TGRL and HDL. Such cholesteryl ester transfer from HDL to TGRL might therefore be negligibly low at low concentrations of LCAT (and low concentrations of HDL, as LCAT is exclusively carried by HDL under our experimental conditions) but might greatly increase at high HDL concentrations, decreasing net cholesterol accumulation in HDL. Reduction of free cholesterol concentration gradient between TGRL and HDL occurring at high HDL concentrations may additionally contribute to the latter effect. Consistent with this notion, the dose-dependence of free cholesterol transfer was characterised by direct relationship with HDL concentration at low concentrations of both UC-HDL and apoB-depleted plasma, most likely reflecting the presence in the assay of increasing concentrations of acceptor HDL particles for TGRL-derived free cholesterol. However, such a direct relationship evolved into an inversed dose-dependence at high concentrations of HDL when apoB-depleted plasma (possessing high CETP activity) was employed as the HDL source. Interestingly, the inverse relationship was not observed in apoB-depleted plasma in the presence of torcetrapib, or in UC-HDL (containing only minor amounts of CETP following ultracentrifugation[29]). In the latter experiment, the transfer of free cholesterol was directly related to HDL concentration throughout the whole concentration range studied, potentially reflecting low CETP activity in the samples.

Clinical value of the assay was assessed in four populations markedly differing in plasma HDL-C. The transfer of free cholesterol to HDL was reduced in low HDL-C patients with AMI, low HDL-C patients with T2D and subjects with extremely high HDL-C levels of >100 mg/dL relative to healthy normolipidaemic controls. When these data were plotted against individual HDL-C levels in all subjects, or against mean HDL-C levels in the groups, inverse U-shape relationships were observed which mirrored the U-shape relationship of overall and cardiovascular mortality with HDL-C concentrations recently reported in large-scale epidemiological studies.[4–6] As a consequence, the relationships of free cholesterol transfer to HDL with cardiovascular and overall mortality obtained from these studies were linear and negative, in clear contrast to the U-shape relationships observed for HDL-C. Albeit circumstantial, these data suggest that free cholesterol transfer to HDL upon TGRL lipolysis by LPL may underlie the U-shape relationship between HDL-C and CVD.

Interestingly, free cholesterol transfer was unchanged in subjects with high HDL-C of 70–100 mg/dL and was only diminished at extremely high HDL-C levels of >100 mg/dL. It is worth noting that the high HDL-C groups expectedly featured a prevalence of female over male subjects, while the AMI group was composed of only males, raising a question of the role of gender for the observed differences. Each of the studied groups was, however, compared with a matched control group and no difference in the TopF transfer was observed between the four control groups despite their differences in the sex ratio, thereby arguing against the role of gender in the between-group effects observed by us.

According to the dose-dependences of HDL capacity to acquire free cholesterol from TGRL upon lipolysis, the decreases in this metric observed in low HDL-C patients with AMI and T2D may straightforwardly reflect low concentrations of acceptor HDL particles. Indeed, no difference in free cholesterol transfer to AMI and control HDL was found when the lipoproteins were compared on a protein concentration basis. Other metabolic alterations potentially underlying decreased free cholesterol transfer in both low and extremely high HDL-C subjects involve elevated CETP and/or LCAT activities, which were, however, not detected by us. Altered HDL particle profile typical of extremely high HDL-C states includes highly elevated levels of large, lipid-rich HDL and reduced concentrations of small, lipid-poor HDL particles.[20,30] As our data document superior capacity of small versus large HDL to acquire free cholesterol upon TGRL lipolysis (which is consistent with the superiority of such particles to efflux cellular cholesterol and to perform other biological functions[31]), the decrease in this metric at extremely high HDL-C might reflect such alterations in the HDL particle profile.

To establish a direct link between lipolytic free cholesterol transfer from TGRL to HDL and development of CVD, we studied postprandial movement of radiolabelled cholesterol in three groups of mice markedly differing in HDL-C. Small amounts of [^{3}H]-cholesterol were consistently found in murine aortic tissue 2 h following oral gavage with this compound. Such aortic accumulation of [^{3}H]-cholesterol was inversely associated with HDL-C concentrations, being highly elevated in low HDL-C apoA-I knock-out animals. In parallel, the capacity of murine HDL to acquire free cholesterol upon TGRL lipolysis by LPL was reduced, resulting in its negative correlation with the aortic accumulation of [^{3}H]-cholesterol. These data additionally attest free cholesterol transfer to HDL upon TGRL

lipolysis by LPL as a negative metric of CVD and directly link HDL metabolism to postprandial atherosclerosis. Interestingly, free cholesterol transfer was not compromised in high HDL-C apoA-I transgenic mice, in contrast to our observations of reduced free cholesterol transfer in human subjects with extremely high HDL-C, potentially reflecting low CETP and LCAT activities in mice.

Together, our data provide evidence that HDL capacity to acquire free cholesterol upon TGRL lipolysis by LPL in vitro, measured using our novel assay, may represent a superior biomarker of cardiovascular risk as compared with plasma HDL-C levels presently employed in clinical practice. The assay is simple, rapid, does not require sophisticated equipment and can be easily run in clinical routine. Moreover, these data support our RRT hypothesis to explain the U-shape association between HDL-C levels and CVD by intravascular free cholesterol transfer from TGRL to HDL in a pathway that can be essential for triglyceride metabolism and energy production. We propose that plasma concentration of HDL-C is an imperfect static measure of cholesterol flux through this dynamic pathway, which is directly associated with the efficacy of cholesterol removal from the circulation only at low-to-normal HDL-C.

Importantly, HDL exerts a plethora of other biological effects, including its capacity to efflux cholesterol from peripheral cells, to protect LDL from oxidative modification, to reduce production of pro-inflammatory phospholipids and to beneficially impact glucose metabolism.[32,33] None of these well-studied activities is, however, known to be reduced at both low and extremely high concentrations of HDL and can thereby account for the complex non-linear relationship between HDL-C levels and CVD. We conclude that this relationship primarily reflects the lipid acceptor role of HDL in the lipolysis.

Author contribution

Designing research studies: MF, MD, ML, LM, EF, FR, CVS, RDS, FB, GB, EG, TH, ACar, EB, MG, PC, PG, PL, WLG, IG, AK. Recruiting study subjects: FR, CVS, RDS, FB, EB, MG, PC, PG. Conducting experiments: MF, MD, ET, ACan, SL, IG, AK. Acquiring data: MF, MD, ET, ACan, ML, LM, SL, FB, CVD, FB, ACar, EB, MG, PC, PG, IG, AK. Analysing data: MF, MD, MP, IG, AK. Writing the manuscript: MF, AK.

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