HDL and Reverse Remnant-Cholesterol Transport (RRT): Relevance to Cardiovascular Disease

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Abstract

Cardiovascular diseases predominantly result from atherosclerosis, a natural biological phenomenon reflecting food intake and energy production in humans. Lipolysis of plasma triglyceride-rich lipoproteins (TGRLs) by lipoprotein lipase is an essential element of energy production that delivers free fatty acids to peripheral cells. High-density lipoprotein (HDL) plays a key role in this process via acquirement of surface lipids, including free cholesterol, released upon TGRL lipolysis. According to the reverse remnant-cholesterol transport (RRT) hypothesis, such removal of cholesterol from remnant lipoproteins followed by transport to the liver with excretion into the bile may represent a major biological function of HDL essential for energy production, which can reduce cholesterol influx in the arterial wall by accelerating removal from circulation of atherogenic TGRL remnants.

HDL: A mystery wrapped in an enigma

Low levels of plasma **high-density lipoprotein-cholesterol (HDL-C**; see Glossary) represent a well-known and widely employed cardiovascular risk factor [1]. Intriguingly, recent large-scale prospective epidemiological studies repeatedly document paradoxically increased cardiovascular and overall mortality at extremely high HDL-C levels (see Clinician's Corner), resulting in the U-shape relationship of the mortality with HDL-C [2-6]. The common belief that the higher HDL-C, the better does not therefore hold for extremely high concentrations [4]. Importantly, the relationship between low HDL-C and cardiovascular disease is typically weakened [2] and can disappear [7] in patients treated with **statins** which presently represent a standard of care.

The relationship between HDL-C and mortality is equally complex in subjects with genetically altered concentrations of HDL-C. Indeed, mutations in the *APOA1*, *ABCA1* and *LCAT* genes result in monogenic low HDL-C disorders which are inconsistently associated with cardiovascular risk and **atherosclerosis** [8]. Moreover, certain mutations resulting in extremely high concentrations of HDL-C, including those in the *SCARB1* and *CETP* genes, paradoxically increase cardiovascular risk [9, 10]. Elevated HDL-C concentrations resulting from alterations in the *CETP* gene are however more consistently associated with reduced cardiovascular disease [11], in parallel with decreased triglyceride, low-density lipoprotein-cholesterol (LDL-C) and **apolipoprotein (apo)** B levels. This observation reflects inherent difficulty of HDL-C epidemiology, which involves associations of altered HDL-C levels with other lipid traits, usually with plasma triglyceride concentrations [8]. In order to define specific contribution of HDL-C to cardiovascular disease, Mendelian randomization is employed using genetic variants that selectively modify HDL-C levels without influencing other lipid traits. This approach does not reveal causal relationships between HDL-C and risk of cardiovascular disease associated with *LIPG* variants [12] or HDL-C genetic scores [12, 13]. However, other studies report that both *APOA1* and *ABCA1* mutations can markedly increase prevalence of cardiovascular disease in subjects with isolated low HDL-C [14].

To explain the association between low HDL-C and cardiovascular disease, the **HDL** hypothesis was developed by Miller and Miller who postulated that enhanced atherosclerosis is due to delayed cholesterol clearance from the arterial wall secondary to reduced HDL levels [15]. An existence of such **reverse cholesterol transport (RCT)** – which contrasts direct cholesterol transport from the liver and the intestine to other tissues - was earlier proposed by

Glomset [16]. The HDL hypothesis subsequently evolved into the RCT hypothesis which included, as major elements, efflux of free cholesterol from macrophages to HDL, esterification of free cholesterol by lecithin:cholesterol acyltransferase (LCAT) with HDL enlargement, and hepatic uptake of cholesteryl ester from HDL followed by its conversion into bile salts which are excreted [17]. The hypothesis assumes that the major atheroprotective function of HDL involves cholesterol efflux from arterial wall cells and that altered metabolism of HDL results in the defective efflux and accelerates atherosclerosis. Such HDL flux hypothesis is primarily based on negative associations of cardiovascular disease with in vitro measurements of cellular cholesterol efflux from lipid-loaded macrophages [17]. HDL equally displays other atheroprotective functions which can be compromised in metabolic diseases associated with low HDL-C and accelerated atherosclerosis [18]. The HDL hypothesis was therefore revised into the HDL function hypothesis which states that atheroprotection is causatively related not to HDL-C but to a cardioprotective HDL function which cannot always be estimated through the HDL-C assay [8, 17].

The major difficulty of the RCT hypothesis includes absence of convincing data documenting removal of cholesterol from human atherosclerotic intima through RCT [19]. Another difficulty involves lack of meaningful relationship between RCT and HDL-C. Indeed, measurements of RCT in humans using labelled cholesterol reveal absence of correlation between tissue cholesterol efflux and HDL-C [20]. This finding agrees with the observation that cholesterol efflux from tissue macrophages provides only a small contribution to HDL-C levels if any [21]. Finally, the RCT pathway can hardly be envisioned to be developed through evolution as cardiovascular disease typically affects humans after their reproductive age [19].

Importantly, the HDL function hypotheses do not provide explanation for the non-linear U-shape association between HDL-C and cardiovascular disease. While HDL function is often deficient at low HDL-C [18], data on the HDL function in subjects with extremely high HDL-C are scarce. Cholesterol movement via the RCT pathway is however unlikely to be deficient in such subjects as cholesterol efflux capacity of their HDL is enhanced, rather than reduced [22, 23]. Interestingly, anti-inflammatory activity of HDL can be deficient in coronary heart disease (CHD) patients with extremely high HDL-C [24]. However, in this study HDL was equally dysfunctional in subjects with normal HDL-C. In addition, angiogenic properties of HDL are weakened at high HDL concentrations [25]. Relevance of this pathway to human atherogenesis however remains to be established.

The classic view of HDL metabolism centred on the RCT pathway has recently been revisited by several studies. Earlier isotopic studies reveal that cholesteryl esters are predominantly delivered to the liver from apoB-containing **lipoprotein**s rather than from HDL in humans [26, 27]. By contrast, free cholesterol is readily taken up from HDL by the liver and secreted in the bile or utilized for bile acid synthesis [26, 27]. Isotopic studies in humans equally demonstrate that the majority of tissue cholesterol efflux occurs via free cholesterol which is rapidly redistributed among plasma lipoproteins [20, 28]. Furthermore, isotopic studies in mice show that while most free cholesterol is rapidly extracted from nascent HDL by the liver via scavenger receptor class B type I (SR-BI), free cholesterol esterification by LCAT is a minor process in HDL metabolism [29]. Most free cholesterol is thereby rapidly transferred from HDL to the liver independent of LCAT activity [29]. Indeed, the flux of free cholesterol is an order of magnitude faster than that of cholesteryl esters [29]. Consistent with these data, endogenous isotopic labelling provides little evidence for progressive enlargement of HDL in humans, revealing that HDL is secreted into the circulation in its entire size distribution from very small to very large particles [30]. In this model, RCT can still be active but mainly within rather than among the HDL sizes [30], primarily involving movement of free cholesterol [29].

With low plasma HDL-C established as cardiovascular risk factor, the concept of therapeutic HDL-C raising gained popularity several decades ago as a novel approach to reduce cardiovascular disease [18]. Emphasis on HDL-C as a therapeutic target however resulted in multiple failures involving cholesteryl ester transfer protein (CETP) inhibitors, fibrates, niacin and other agents. CETP inhibitors potently increase HDL-C and less potently decrease LDL-C, triglycerides and apoB, exerting a spectre of clinical effects which vary from deleterious off-target to modestly beneficial [31]. Fibrates and niacin moderately raise HDL-C, more potently reduce triglycerides and can decrease cardiovascular risk in patients not treated by statins [32]. Subgroup analysis suggests that statin-treated patients with elevated triglyceride levels and low HDL-C might still benefit from these agents [33].

The negative results of HDL-C-raising trials can potentially be accounted for by excessive HDL-C–raising, consistent with elevated mortality observed at extremely high HDL-C in epidemiological studies [2-6]. The absence of clinical benefit may equally reflect lack of beneficial effect on atheroprotective HDL functions. However, HDL from subjects treated with CETP inhibitors or niacin display enhanced, rather than reduced, cholesterol efflux capacity from macrophages [31, 34].

It is of key importance that all modern large-scale clinical trials of HDL-C-raising agents were performed in patients treated by statins. In contrast to earlier trials carried out in the pre-statin era, HDL-C-raising on a background of statin treatment did not exert expected beneficial effects on cardiovascular disease [32]. Indeed, HDL-C-raising drugs were only efficient at high baseline LDL-C [35].

Together, epidemiology, biological function, metabolism and therapeutic targeting of HDL are difficult to reconcile within the existing concepts. As lipoprotein metabolism represents an intricate network of pathways linking different lipoprotein classes, a broader look at HDL in the context of its relationships to other lipoproteins, primarily to those rich in triglycerides, may allow revisiting our view of this enigmatic particle. More specifically, closer analysis of the role of triglycerides in cardiovascular disease unexpectedly proves useful to resolve the HDL controversy.

Metabolic links between HDL and TGRL: The mystery is opening

Non-lipolytic and lipolytic pathways

Intravascular HDL metabolism is intimately linked to that of **triglyceride-rich lipoproteins (TGRL)**, a phenomenon which is frequently manifested as a negative correlation between plasma levels of HDL-C and triglycerides [36, 37]. In the circulation, HDL and TGRL interact via multiple metabolic pathways a most studied of which includes heteroexchange of core lipids mediated by CETP. Plasma CETP primarily mediates heteroexchange of cholesteryl esters and triglycerides between spherical HDL and TGRL, resulting in the formation of triglyceride-enriched HDL and cholesteryl ester-enriched TGRL.

Another key, but frequently overlooked, pathway linking HDL and TGRL involves transfer to HDL of surface remnants generated during TGRL lipolysis by **lipoprotein lipase (LPL)** [28, 38]. Lipolysis of TGRLs is an essential element of energy production in mammals aimed at supplying peripheral tissues with free fatty acids. This 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 [39]. When TGRLs (chylomicrons and very low-density lipoprotein (VLDL)) enter blood circulation, their triglycerides are lipolysed to free fatty acids by LPL on the surface of the endothelium with a shrinkage of the hydrophobic lipoprotein core and production of smaller-size, higher-density remnant TGRL also called core remnants [40-42]. Excess molecules of the surface monolayer surrounding the

hydrophobic core are shed from the particles in a form of surface remnants; such molecules include surface apolipoproteins, phospholipids and free cholesterol. Most of the excess material comes off as unstable bilayer sheets which can form large-size vesicular structures [43]. In addition, small discoid pre-beta HDL particles can be assembled directly from apolipoproteins and surface lipids of TGRL [41].

Mass transfer to HDL during lipolysis

The surface remnants of TGRL predominantly fuse with HDL, adding material to plasma HDL pool which thereby helps converting remnants to new HDL particles [43]. LPL-mediated lipolysis is a rate-limiting step of TGRL catabolism. Both LPL activity and fractional catabolic rate of VLDL apoB and triglycerides are positively correlated with plasma HDL-C, revealing that HDL-C is directly associated with the removal rate of postprandial lipoproteins from human circulation [28, 38, 39]. Variations in LPL activity may account for a considerable portion of the variability in HDL-C levels, indicating that HDL-C represents an index of LPL activity and a biomarker of TGRL lipolysis by LPL [38, 44].

HDL can readily acquire both polar (free cholesterol, phospholipid) and non-polar (cholesteryl ester, triglyceride) lipids. The potent lipid-acquiring properties underlie efficient lipid transfer to HDL during TGRL lipolysis. In vitro experiments extensively document transfer of surface components, including free cholesterol, phospholipid and apolipoproteins A-I, A-II, C-II, C-III and E, from TGRL to HDL upon lipolysis [28, 40, 42, 43, 45, 46]. Such transfer can be traced using isotopic technique, demonstrating that in subjects receiving radiolabelled cholesterol orally, radioactivity peak in TGRL precedes that in HDL [47].

Formation of large HDL. Addition of LPL to serum results in the accumulation of large, light HDL2 particles paralleled by a decrease in small, dense HDL3 in vitro [48]. The size of the acceptor HDL particles is increased following TGRL lipolysis, whereas their density is reduced [38, 40, 43].

Similarly, HDL reveals enlarged size, elevated molecular mass and diminished density after fat meal in vivo [49, 50]. Levels of large, light HDL2 are increased, while those of small, dense HDL3 can be either increased or slightly decreased after fat meal [49, 50]. The content of many constituents, including phospholipid, protein (primarily **apoA-I**), free cholesterol, cholesteryl esters and triglycerides, can be elevated in HDL2 and HDL3 after fat meal [49, 50], although cholesteryl esters are typically not accumulated in postprandial HDL and can even be depleted, resulting in a null effect on HDL-C.

Underlying mechanisms involve transfer of surface constituents from TGRL predominantly to HDL3, which is enlarged to become HDL2 [49], or to both HDL2 and HDL3 [50]. Small, dense, lipid-poor HDL3 particles represent preferential acceptors for surface lipids released from TGRL during lipolysis [51]. Interestingly, small pre-beta HDL containing apoA-I and phospholipid can be released from HDL as a result of the transfer of surface material from TGRL during lipolysis [38]. In this process, spherical HDL combined with the surface remnants grows in size, releasing pre-beta HDL [52]. Transfer of phospholipid and free cholesterol to HDL3 enhances LCAT-mediated formation of cholesteryl esters, facilitating conversion of HDL3 into HDL2 [50]. As a result, there is a precursor-product relationship between TGRL surface components and HDL2 mediated by HDL3 [38]. High plasma HDL concentrations can therefore be both a result and a cause of efficient clearance of plasma TGRL as occurs in females vs. males, alcohol users vs. non-users, and upon physical activity [38].

Molecular mechanisms. Torcetrapib, specific CETP inhibitor, enhances accumulation of fluorescent cholesterol in HDL upon TGRL lipolysis by LPL [51], probably reflecting inhibition of CETP-mediated transfer of cholesteryl esters from HDL to TGRL following esterification of free cholesterol by LCAT. In support of this mechanism, transfer of cholesteryl esters from HDL to VLDL is enhanced when CETP is added to the mixture of VLDL, HDL and LPL [53]. Furthermore, LCAT inhibition by iodoacetamide increases accumulation of fluorescent cholesterol in HDL upon TGRL lipolysis [51], consistent with the multi-step mechanism of free cholesterol movement between HDL and TGRL upon LPL-induced lipolysis in which LCAT acts in concert with CETP to promote removal of cholesterol from HDL back to TGRL (as well as to LDL) following its esterification. Such continuous flow of cholesterol through HDL mediated by LCAT and CETP may underlie the observation of largely stable concentrations of HDL-C in the postprandial phase [49, 50].

Formation of TGRL remnants. Presence of HDL facilitates TGRL lipolysis, removing lipids and proteins released during this process [43]. Lipolysis of human VLDL by LPL in the presence of HDL results in accelerated decrease in VLDL content of cholesterol and formation of both VLDL core and surface remnants [43]. The surface remnants form liposome-like vesicles in the presence of low levels of HDL and are assimilated into HDL to form larger HDL particles in the presence of excess HDL [54]. Such acquirement of surface TGRL remnants may represent a key biological function of HDL which may prevent their accumulation in the arterial wall as originally proposed by Chung et al. [43, 54]. In addition,

the removal of excess surface remnants by HDL can facilitate hepatic uptake of core remnants [55].

Role of apoA-I. ApoA-I represents a typical amphipathic protein which binds avidly to lipids [56, 57]. Such properties render apoA-I a potent biological detergent which interacts with minimal amounts of lipids present in aqueous phase, instantly transforming them into highly organised, stable, micellar lipoprotein complexes in the HDL density range [56, 57]. Solubilisation of lipids (e.g. upon cholesterol efflux from peripheral cells or across small intestine) may therefore represent a major function of apoA-I [58]. Other biological activities of apoA-I, including its potent anti-inflammatory and antioxidative properties [18], can similarly reflect binding of pro-inflammatory and oxidised lipids [24]. In addition, apoA-I forms complexes with proteins displaying prominent anti-atherosclerotic and anti-inflammatory properties, such as paraoxonase 1, further enhancing atheroprotection [59].
The liver is traditionally thought of to represent the major site of apoA-I production. However, apoA-I is equally synthesised actively throughout the small intestine. Upon production, apoA-I is secreted by the intestine into the lymph in a process which is central for the metabolism of exogenous lipids. Together with the liver, the ileum is a principal source of newly synthesized apoA-I [60]. ApoA-I synthesis and secretion into the lymph increase with lipid absorption [61]. Intestinal apoA-I thereby provides a major contribution to plasma apoA-I levels, which can reach 30-40% of total plasma apoA-I supply in humans [61]. In rats, intestine produces over 50% of total apoA-I, with the liver producing the rest [62].
The intestine secretes apoA-I as a part of two lipoprotein species, chylomicrons and discoid nascent HDL [60, 61]. Chylomicron-associated apoA-I accounts for 60-80% of total apoA-I in lymph [63]. Intriguingly, apoA-I prevails among apolipoproteins of chylomicrons, while apoB is a quantitatively minor component (3.4 wt% of total protein in humans) [61]. In intestinal lymph, apoA-I accounts for 15-35 wt% of total chylomicron protein, with apoB and apoA-IV accounting for 10% each, while the contribution of apoCs prevails at 42-50% [41, 64]. ApoA-I is also a major protein component of chylomicrons from mesenteric lymph in rats (56 wt%) [61]. Remarkably, the apolipoprotein composition of chylomicrons resembles that of HDL [61].
Following secretion, most of chylomicron apoA-I is transferred to HDL during lipoprotein catabolism. Similar transfers occur when chylomicrons are incubated with plasma in vitro [65]. The main reason why apoA-I needs to be removed from TGRLs upon their lipolysis

appears to involve the necessity to pick up surface lipids released during this process, leading to the formation of HDL [54].

TGRLs: Unravelling the enigma

Epidemiology and genetics

Triglycerides. Association of high triglyceride levels with cardiovascular risk has recently been established in large-scale epidemiological studies using multivariate models [37, 66]. Genetic evidence obtained in Mendelian randomization studies supports raised concentrations of triglycerides being causally related to cardiovascular and all-cause mortality [36]. Specific genetic mechanisms that affect the LPL pathway, increase plasma triglycerides and augment cardiovascular risk include loss of function of LPL and gain of function of apoC-III, apoA-V and ANGPTL4 [37, 67].

Complete LPL deficiency is not considered atherogenic in humans. Indeed, extremely large size of TGRL particles accumulated in this condition precludes their entry in the arterial wall [36]. However, partial LPL deficiency is associated with elevated plasma triglycerides, reduced HDL-C and increased odds of CHD, while gain-of-function LPL variants are cardioprotective [68]. By contrast, mutations of APOC3 result in decreased plasma apoC-III, triglycerides, apoB and LDL-C, increased HDL-C and reduced CHD risk [66, 67]. Mechanistically, the association between apoC-III and cardiovascular disease primarily reflects inhibitory effects of the protein towards triglyceride lipolysis and hepatic uptake of TGRL remnants [37] and may reflect deleterious effects on HDL function [69].

Remnant cholesterol. Circulating TGRLs are not directly atherogenic and become deleterious upon their conversion to smaller-size remnant particles [36]. Such TGRL remnants are enriched in cholesterol, carrying 5 to 50 times more cholesterol per particle than LDL [37] and accounting for up to one-third of total plasma cholesterol [70]. As a result, **remnant cholesterol** can be even more atherogenic than LDL-C [71]. While remnant cholesterol is often calculated using LDL-C according to the Fridewald's formula, resulting in a direct relationship with plasma triglycerides, the association with cardiovascular disease holds when remnant cholesterol is measured directly [72]. Because triglycerides can be degraded by most cells, but cholesterol cannot be degraded by any, cholesterol content of TGRLs is more likely to be the cause of atherosclerosis and cardiovascular disease than raised

triglycerides [36]. High triglyceride concentrations can therefore be considered as a marker for high concentrations of TGRL remnants rich in cholesterol [36].

As plasma levels of remnant cholesterol parallel those of triglycerides and the latter are inversely correlated with HDL-C, remnant cholesterol is equally correlated negatively with HDL-C [72, 73], indicating that low HDL-C can be regarded as a biomarker of both high triglycerides and high remnant cholesterol levels [36, 73]. As mutual adjustments can eliminate associations with CHD of HDL-C and TG [74], these lipid traits may influence cardiovascular disease through the same processes.

Metabolism

Altered metabolism of TGRL particles results in the accumulation in the circulation of TGRL remnants which can be cytotoxic, pro-inflammatory and pro-atherogenic, inducing cholesterol accumulation in arterial wall macrophages [37, 75]. In support of this notion, electron microscopy reveals that atherosclerotic lesions in both humans and experimental animals contain numerous, large, extracellular, liposome-like particles, which differ from extracellular VLDL- and LDL-like particles and intracellular lipid droplets [54]. Such in vivo derived particles are similar to liposome-like lipolytic surface remnants of TGRL produced during in vitro lipolysis [54]. Moreover, early post-mortem human atherosclerotic plaques contain a large part of cholesterol in a form of liposome-like particles [54]. Consistent with these data, such plaques contain numerous particles rich in free cholesterol but poor in cholesteryl esters, which are present before foam cells start to accumulate [76]. In addition, atherosclerotic lesions present with large amounts of non-lipoprotein-associated lipid which can originate from lipolysis [77].

Therapeutic targeting

Plasma triglyceride levels can be decreased by supplementation with polyunsaturated fatty acids. When statin-treated patients possessing elevated triglycerides received 2 g of icosapent ethyl twice daily in the REDUCE-IT trial, the risk of ischemic events, including cardiovascular death, was decreased by -25% relative to placebo paralleled by a decrease in triglycerides of -18.3% [78]. In addition, reduction of apoC-III is associated with improved lipoprotein profile and can be considered as a promising approach to reduce cardiovascular disease via triglyceride lowering [79]. Therapeutic modulation of the LPL pathway thereby bears a potential to reduce both triglyceride levels and the risk of CHD.

RRT hypothesis: The mystery solved?

The hypothesis

Computed tomographic findings of atherosclerosis in ancient cultures [80] together with fatty streak formation in human fetal aortas [81] reveal that human atherosclerosis is a natural process. A natural way of atherogenesis was originally described by Moreton who proposed that lipid particles are retained and deposited in the arterial intima from the plasma-derived nutrient lymph stream to be phagocyted by macrophages with the formation of foam cells [82]. Later developed by Zilversmit [83], the concept of atherosclerosis as a postprandial phenomenon is supported by recent epidemiological findings of the association of elevated levels of postprandial triglycerides and remnant TGRL with cardiovascular disease [36]. Together with the data on natural history of atherosclerosis, this concept can be interpreted in such a way that atherogenesis represents an undesirable, but inevitable, consequence of food intake and energy production, being a common biological phenomenon in humans.

According to the widely accepted 'response-to-retention' model of atherosclerosis [84], retention of cholesterol-rich, apoB-containing **remnant lipoproteins** within the arterial wall is the key initiating event in atherogenesis. The presence of HDL allows eliminating from circulation both surface and core remnants of TGRL which can otherwise accumulate in the arterial wall and induce atherosclerosis [43]. Given its presence in lower vertebrates and predominance in most species, HDL must play an important biological role from an evolutionary standpoint and can be important for survival [19]. The central place of HDL in the postprandial lipid metabolism and energy production provides an excellent example of such a role, which can be particularly important in small animals, such as rodents, presenting with elevated energy requirements and accelerated lipid metabolism in the postprandial state. The atheroprotective role of HDL can thereby be secondary to its role in TGRL lipolysis, reflecting its capacity to indirectly, but potently, reduce cholesterol influx in the arterial wall by ensuring proper removal from circulation of cholesterol-rich TGRL remnants. This pathway exemplifies the long-sought mechanism underlying HDL-mediated protection from cardiovascular disease, which both provides a major contribution to HDL-C levels and maintains low levels of cholesterol in the artery wall [85].

Steady-state concentrations of HDL-C may represent a biomarker of the removal of remnant TGRL cholesterol through the plasma compartment. Indeed, transfer of free cholesterol from TGRL upon LPL-induced lipolysis represents a major source of HDL-C in humans [38, 44].

Importantly, the contribution of this pathway to HDL-C exceeds that provided by cholesterol efflux from all extra-hepatic tissues combined, and even more so from arterial wall cells [86]. Mechanistically, free cholesterol acquirement by HDL upon TGRL lipolysis can primarily be mediated by lipoprotein complexes containing apoA-I which ensures proper structure and function of HDL particles essential for shuttling excess surface lipids through the bloodstream [56, 57]. Formation of such complexes may represent a key biological role of intestinally-derived, chylomicron-associated apoA-I [64]. While apoA-I can solubilise excess surface lipids released from TGRL upon lipolysis, HDL phospholipid may provide lipid surface required for their absorption [41, 43, 54].

Together, these considerations can be combined within a **reverse remnant-cholesterol transport (RRT)** hypothesis (Box 1) which involves acquirement by plasma HDL of surface remnants, including free cholesterol, of TGRL upon intravascular lipolysis by LPL with subsequent transport of remnant-derived cholesterol to the liver in a pathway which originates in the intestine with the secretion of apoA-I and cholesterol in chylomicrons followed by their transport to plasma via lymph (Figure 1). Remnant-derived free cholesterol can thereby be esterified under the action of LCAT before being transferred from HDL to the liver either directly via SR-BI or through apoB-containing lipoproteins and their hepatic receptors, though the both pathways can handle both free and esterified cholesterol [29, 87]. The latter pathway can quantitatively prevail in humans [26, 27], delivering cholesterol from HDL to apoB-containing particles via both CETP-dependent and independent mechanisms [88].

The RRT hypothesis implies that lipolytic free cholesterol transfer from TGRL to HDL is impaired under metabolic conditions associated with cardiovascular disease, leading to the accumulation of remnant-derived cholesterol in the arterial wall (Figure 2). Available data indeed demonstrate that the rate of this process can be diminished at both low and extremely high HDL-C, consistent with the U-shape epidemiology (Box 1). Such impaired free cholesterol transfer to HDL upon lipolysis may result from decreased levels of acceptor HDL particles, their altered profile and composition as well as from alterations in lipid transport proteins and enzymes, and is frequently accompanied by elevated levels of triglycerides (Box 2).

Therapeutic corollary

The RRT hypothesis allows revisiting our view of therapies modulating HDL metabolism. The outcome of HDL-C-raising approaches was proposed to depend on the mechanism by

which HDL-C is raised [89]. According to the RRT hypothesis, HDL-C-raising can be cardioprotective when it accelerates removal from circulation of atherogenic TGRL remnants and is accompanied by a reduction of remnant cholesterol (Figure 2). Rather than enhancing cholesterol efflux from macrophages as proposed by the RCT model, novel approaches should focus at diminishing cholesterol influx into macrophages from remnant lipoproteins. Such concept can be considered preventive, in contrast to the curative approach of the enhanced RCT.

LPL pathway. As LPL pathway provides major contributions to circulating levels of both HDL-C and triglycerides, regulation of this pathway appears most promising in the framework of RRT. Indeed, HDL-C levels can be efficiently raised by LPL activation in parallel to decreasing plasma triglycerides [90]. Experiments with apoC-III antisense oligonucleotides reveal sustained reductions in plasma triglycerides, VLDL-cholesterol, VLDL apoB, apoB-48, VLDL particle number and plasma apoC-III in vivo [91]. In parallel, HDL-C is elevated in CETP-expressing species [79].

ApoA-I synthesis. Metabolic states in which apoA-I levels are high can be expected to result in efficient removal of surface lipids from TGRL undergoing lipolysis, fostering efficient triglyceride catabolism and HDL formation [92]. Other biological activities of apoA-I can further contribute to cardioprotection [18]. Interestingly, epidemiological data suggest that apoA-I may represent a more relevant therapeutic target as compared to HDL-C [89].

Statins. Statin treatment eliminates, or greatly reduces, potential therapeutic benefits of HDL-C-raising in cardiovascular disease [32]. Acting via enhanced hepatic expression of LDL receptors, statins can accelerate removal of atherogenic core TGRL remnants in a pathway which may overlap with that stimulated by HDL. Benefits of statins can be derived from both LDL-C lowering and HDL-C raising [93], with the latter potentially reflecting enhanced free cholesterol flux through the lipolytic pathway. Consistent with this possibility, low baseline HDL-C and high baseline triglycerides are strong, independent predictors of high HDL-C elevation induced by statins [94].

Concluding remarks

The RRT hypothesis provides a framework for revisiting our present view of HDL metabolism, incorporating unexpected and paradoxical results of recent large-scale trials and

epidemiological studies. Inevitably leaving numerous questions open (see Outstanding Questions), the hypothesis links the complex U-shape relationship between HDL-C and cardiovascular disease [2-6] to free cholesterol transfer to HDL upon TGRL lipolysis by LPL [51]. Indeed, inverse U-shape relationships between the latter functional metric and HDL-C result in linear and negative relationships of free cholesterol transfer to HDL with cardiovascular and overall mortality obtained from epidemiological studies, in clear contrast to the U-shape relationships observed for HDL-C [51].

From a diagnostic point of view, HDL capacity to acquire free cholesterol upon TGRL lipolysis by LPL in vitro may provide a superior biomarker of cardiovascular risk as compared to plasma HDL-C levels [51]. Plasma concentration of HDL-C can therefore be considered as an imperfect static measure of cholesterol flux through this dynamic pathway, which is directly associated with the efficacy of cholesterol removal from circulation only at low-to-normal HDL-C. The lipolytic FC transfer from TGRL to HDL can however still be limited in its capacity to predict cardiovascular risk as suggested by the presence of moderate hypertriglyceridemia and absence of accelerated atherosclerosis in some forms of low HDL-C **dyslipidemia**s, such as in carriers of apoA-I Milano [8]. Another weakness of the RRT hypothesis that requires further studies involves absence of clear links to inflammatory and infectious diseases.

Although HDL exerts a plethora of other biological activities, none of them is known to be reduced at both low and extremely high concentrations of HDL and can thereby account for the U-shape relationship between HDL-C levels and cardiovascular disease. This relationship may therefore primarily reflect the lipid acceptor role of HDL in lipolysis. Novel therapies targeting HDL metabolism should therefore focus primarily at the acceleration of lipolytic cholesterol fluxes through the RRT pathway.

Figure legends

Figure 1: Reverse remnant-cholesterol transport (RRT) pathway. (A) Upon triglyceride (TG) lipolysis by LPL, triglyceride-rich lipoproteins (TGRL) are converted into core and surface remnants. Core remnants are cleared by hepatic LDL receptors (LDL-R). Surface remnants contain free cholesterol (FC), phospholipid (PL) and apolipoproteins (apos) which combine with the plasma HDL pool, resulting in FC esterification by LCAT and enlargement of HDL particles. Their FC and cholesteryl ester (CE) are transported to the liver directly via SR-BI (in green) or indirectly via apoB-containing lipoproteins. The traditional reverse cholesterol transport (RCT) pathway from the arterial wall is shown as dotted lines. Lipoprotein components are in orange, and enzymes and lipid transfer proteins in blue. (B) ApoA-I is secreted by the intestine as a constituent of chylomicrons which are transported to the blood through the lymph and form a pool of plasma TGRL, together with hepatically produced very low-density lipoprotein (VLDL).

Figure 2: Atherosclerosis and potential sites of therapeutic intervention. Free cholesterol transfer from plasma triglyceride-rich lipoproteins (TGRL) to high-density lipoproteins (HDL) can be impaired (red circles) when (1) TGRL lipolysis is inhibited (LPL deficiency, gain-of-function mutations of apoC-III), (2) levels of acceptor HDL particles are reduced (CHD, AMI, Type 2 diabetes, apoA-I deficiency, ABCA1 deficiency), (3) the particles are already enriched in FC (extremely high HDL-C), and/or (4) cholesterol transfer under the action of CETP and LCAT from HDL back to TGRL is enhanced (elevated CETP activity). Clearance of remnant cholesterol is delayed and the remnants can enter the arterial wall (red dotted arrows), deposit cholesterol and cause atherosclerosis. Potential sites of therapeutic intervention (green circles) to accelerate cholesterol removal through the RRT pathway include activation of TGRL lipolysis by LPL (a) and enhanced apoA-I production (b).

Glossary

Apolipoprotein (apo): Protein that binds and transports lipids in a form of lipoproteins in the circulatory and lymphatic systems; target lipids to receptors at sites of degradation, storage, transformation and recycling

Apolipoprotein A-I (apoA-I): Potent biological detergent and major apolipoprotein of HDL that ensures structure of HDL particles and endows them with specific biological functions which primarily involve lipid binding

Atherosclerosis: Pathological process that typically underlies cardiovascular morbidity and mortality via formation of atherosclerotic plaques, resulting from the progressive accumulation of cholesterol and other lipids in native and oxidized forms, extracellular matrix and inflammatory cells, in the intima and media of the arterial wall

Dyslipidemia: A disorder of lipoprotein metabolism, which may include specific lipoprotein overproduction or deficiency

High-density lipoprotein (HDL): A class of cholesterol-rich lipoprotein particles which are small, dense, lipid-poor and protein-rich as compared to other lipoproteins of human plasma. Human HDL particles are typically spherical with a diameter of 7-12 nm, molecular mass of 80-450 kDa and density of 1.063-1.21 g/ml

High-density lipoprotein-cholesterol (HDL-C): Cholesterol carried by HDL. Low levels of plasma HDL-C are associated with cardiovascular disease and are widely used as a cardiovascular risk factor in clinical practice. Cholesterol carried by HDL is colloquially referred to as “good cholesterol”

Lipoprotein: Plurimolecular, quasi-spherical, pseudomicellar complex composed of lipids solubilised by proteins possessing specialised structure and function, the apolipoproteins

Lipoprotein lipase (LPL): Key enzyme of lipoprotein metabolism present on the blood endothelium to ensure lipolysis of triglycerides in TGRL with a release of free fatty acids

Remnant cholesterol: Cholesterol carried by remnant TGRLs

Remnant lipoproteins (also termed remnant TGRL): Native TGRLs partially lipolysed by LPL. Include chylomicron remnants and VLDL remnants, such as intermediate-density lipoprotein (IDL)

Reverse cholesterol transport (RCT): Transfer of cholesterol from peripheral cells to the liver for excretion into the bile

Reverse remnant-cholesterol transport (RRT): Transfer of cholesterol from TGRL remnants to HDL upon LPL-induced lipolysis followed by transport of the remnant-derived cholesterol to the liver for excretion into the bile

Statins: Class of lipid-lowering drugs that inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme of hepatic cholesterol biosynthesis, decrease plasma levels of low-density lipoprotein-cholesterol (LDL-C) and reduce cardiovascular mortality

Triglyceride-rich lipoprotein (TGRL): Large, light, lipid-rich and protein-poor lipoproteins of intestinal (chylomicrons) and hepatic (very low-density lipoprotein and intermediate-density lipoprotein) origin. TGRLs predominantly carry triglycerides which are lipolysed under the action of LPL to release free fatty acids taken up by tissues as a source of energy. TGRLs are therefore essential for energy production

Clinician's Corner

- Low plasma levels of HDL-C represent a well-known and widely employed cardiovascular risk factor. Both cardiovascular and overall mortality are however increased at extremely high HDL-C levels, resulting in the U-shape relationship with HDL-C. Therapeutic HDL-C-raising in patients treated with statins resulted in multiple failures involving CETP inhibitors, fibrates, niacin, reconstituted HDL infusions and other approaches

- Lipolysis of TGRLs is an essential element of energy production. Elevated concentrations of triglycerides are causally associated with cardiovascular risk and represent biomarkers of atherogenic, cholesterol-rich remnant TGRL. Altered metabolism of TGRL results in the remnant accumulation in the arterial wall and atherosclerosis, a natural process which represents undesirable, but inevitable, consequence of TGRL lipolysis, food intake and energy production. A key pathway linking HDL and TGRL involves transfer to HDL of surface, cholesterol-containing remnants generated during TGRL lipolysis by lipoprotein lipase (LPL). Acquirement of surface lipids released upon TGRL lipolysis by LPL may represent a key biological function of HDL essential for energy production. The atheroprotective function of HDL can be secondary to its role in TGRL lipolysis, indirectly reducing cholesterol influx in the arterial wall by accelerating removal from circulation of cholesterol-rich TGRL remnants

- The reverse remnant cholesterol transport (RRT) hypothesis explains the U-shape relationship between plasma HDL-C levels and cardiovascular disease by impaired transfer of cholesterol to HDL from TGRL upon their lipolysis by LPL in subjects with both low and extremely high HDL-C. Plasma concentration of HDL-C can be regarded as an imperfect static measure of remnant cholesterol removal through this dynamic pathway, which is only clinically relevant at low HDL-C levels. HDL capacity to acquire free cholesterol upon TGRL lipolysis by LPL in vitro may represent a superior biomarker of cardiovascular risk as compared to plasma HDL-C

- HDL-C-raising can be cardioprotective when it accelerates removal from circulation of remnant cholesterol. Rather than enhancing cholesterol efflux from macrophages as

proposed by the reverse cholesterol transport (RCT) model, the RRT hypothesis proposes to focus at diminishing cholesterol influx in the arterial wall from remnant lipoproteins. Such concept can be considered preventive, in contrast to the curative approach of the enhanced RCT

Text Box 1

The RRT hypothesis

Reverse remnant-cholesterol transport (RRT) hypothesis explains the relationship between plasma HDL-C levels and cardiovascular disease by impaired transfer of free cholesterol to HDL from surface remnants of TGRL upon their lipolysis by LPL with subsequent transport of remnant-derived cholesterol to the liver.

This pathway originates in the intestine with the secretion of apoA-I and cholesterol in chylomicrons followed by their transport to plasma via lymph. According to this concept, plasma concentrations of HDL-C represent an imperfect static measure of cholesterol flux through this dynamic pathway.

Evidence supporting the hypothesis

Low HDL-C

Transfer of free cholesterol to HDL from TGRL upon lipolysis by LPL is decreased (-45% vs controls) in low HDL-C patients with **acute myocardial infarction** (AMI) [51]. This finding may reflect impaired removal of remnant cholesterol from TGRL during lipolysis in the postprandial phase. Indeed, postprandial response to fat load is abnormally elevated in survivors of AMI who display elevated postprandial triglycerides relative to controls [55]. Furthermore, postprandial response of AMI patients to exogenous fat is characterised by decreased HDL-C, consistent with abnormal free cholesterol transfer [95]. In addition, postprandial increases in HDL free cholesterol and phospholipid are diminished in CHD patients relative to controls upon fat load [96].

Patients with **LPL deficiency** display low HDL-C, in addition to severe hypertriglyceridemia [97]. Furthermore, circulating triglyceride concentrations are highly elevated and HDL-C is strongly decreased in atherosclerotic LPL -/- mice [98]. Reductions in HDL-C are also observed upon infusions of anti-LPL antibodies in experimental animals [99].

Postprandial triglyceride response is markedly exaggerated in low HDL-C subjects with **apoA-I deficiency** caused by an autosomal apo A-I Q[-2]X mutation who feature premature CHD [100]. Furthermore, postprandial accumulation in murine aortic tissue of [^{3}H]-cholesterol is highly elevated in apoA-I knock-out mice [51]. In parallel, the capacity of

murine HDL to acquire free cholesterol upon TGRL lipolysis by LPL is reduced and negatively correlated with the aortic accumulation of [^{3}H]-cholesterol [51].

Plasma triglyceride levels are elevated in homozygous ATB-binding cassette transporter A1 (**ABCA1) deficiency** [101]. Mechanism underlying the hypertriglyceridemia may involve delayed clearance of TGRL components through HDL. Indeed, ABCA1 may function to ensure HDL maturation and maintenance of plasma HDL pool required for efficient acquirement of surface remnants from TGRL [102].

Extremely high HDL-C

The transfer of free cholesterol to HDL upon TGRL lipolysis is reduced in subjects with extremely high HDL-C levels of >100 mg/dl relative to normolipidemic controls but unchanged in subjects with high HDL-C of 70 to 100 mg/dl [51]. Consistent with the latter, the free cholesterol transfer is not compromised in high HDL-C apoA-I transgenic mice, potentially reflecting low CETP and LCAT activities in mice [51].

Text Box 2

Determinants of impaired free cholesterol transfer to HDL from TGRL upon lipolysis

Levels of acceptor HDL particles

Dose-dependences of lipolytic free cholesterol transfer to HDL reveal direct relationships with HDL concentrations at low-to-normal concentrations [51], most likely reflecting the presence in the assay of increasing amounts of acceptor HDL particles. As a corollary, decreases in the free cholesterol transfer observed in low HDL-C patients with AMI and Type 2 diabetes may straightforwardly reflect low concentrations of the HDL acceptors [51]. Mechanistically, such deficiency can limit the amount of surface lipids which can be acquired by HDL.

Profile of acceptor HDL particles

HDL particles are highly heterogeneous [103, 104]. Small, dense, lipid-poor and protein-rich HDLs are potent in their biological activities, including the capacity to acquire free cholesterol upon TGRL lipolysis by LPL [51]. Extremely high HDL-C states feature elevated levels of large, lipid-rich HDL and reduced concentrations of small, lipid-poor HDL particles [105, 106]. The superior capacity of small vs. large HDL to acquire free cholesterol upon TGRL lipolysis may therefore contribute to reduced free cholesterol transfer to HDL observed in subjects with extremely high HDL-C levels [51].

Lipid composition of HDL

HDL particles enriched of free cholesterol acquire less free cholesterol as compared to those depleted of this lipid [107]. Both human subjects and mice displaying high HDL-C levels, such as those with SR-BI deficiency, possess enlarged, free cholesterol-enriched HDL [29, 108]. HDL enrichment in free cholesterol may thereby contribute to defective free cholesterol removal from TGRL upon lipolysis in subjects with extremely high HDL-C [51]. In contrast, low content of free cholesterol [104] may contribute to the potent capacity of small, dense HDL3 to acquire free cholesterol from lipolysed TGRL [51].

Lipid transfer proteins and enzymes

Both CETP and LCAT play inhibitory roles towards cholesterol accumulation to HDL upon lipolysis, acting along the same pathway of free cholesterol esterification and removal from HDL in a form of cholesteryl esters [51]. Given a rate-limiting role of LCAT for this process [27], such cholesteryl ester transfer from HDL to TGRL might be negligibly low at low concentrations of LCAT (and low concentrations of HDL) but might greatly increase at high HDL concentrations, decreasing net cholesterol accumulation in HDL [51].

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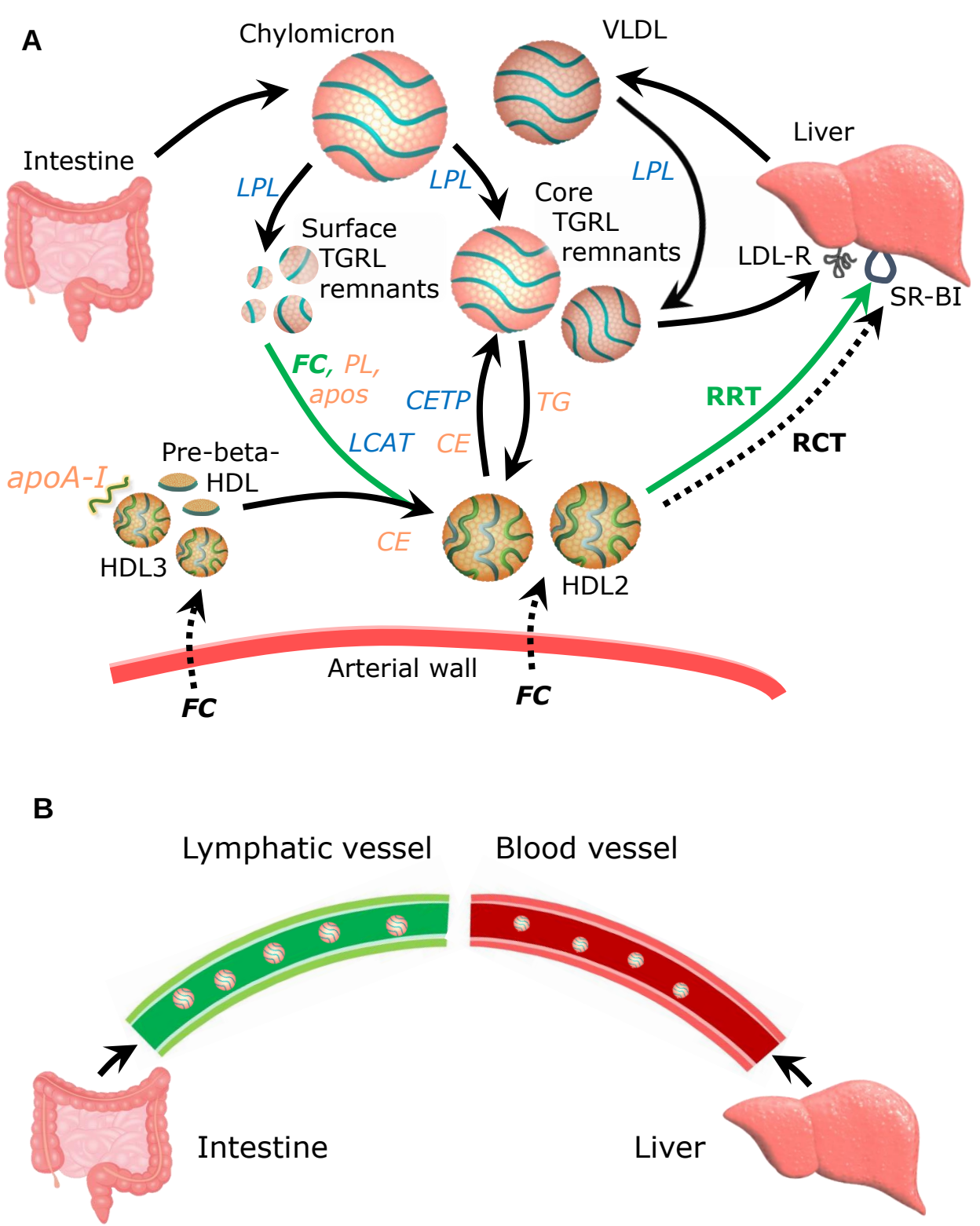
A
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VLDL
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Core
TGRL
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LDL-R
SR-BI
FC, PL,
apos
CETP
TG
RRT
RCT
LCAT
CE
apoA-I
Pre-beta-
HDL
HDL3
CE
HDL2
Arterial wall
FC
FC
B
Lymphatic vessel
Blood vessel
Intestine
Liver

Figure 1

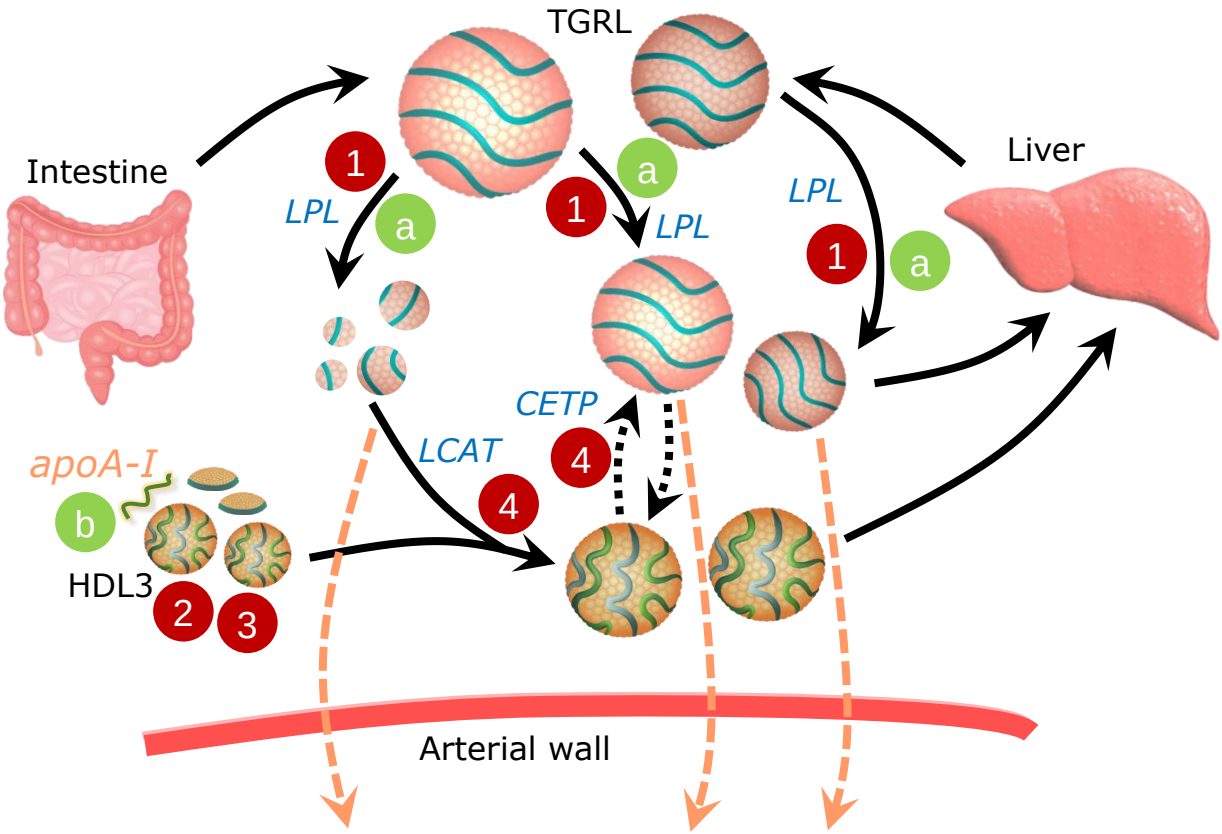
TGRL
Intestine
Liver
1
LPL
a
1
a
LPL
LPL
1
a
apoA-I
b
HDL3
2
3
CETP
4
LCAT
4
Arterial wall

Figure 2