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HDL AND REVERSE CHOLESTEROL TRANSPORT IN HUMANS AND ANIMALS: LESSONS FROM PRE-CLINICAL MODELS AND CLINICAL STUDIES

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Abstract

The ability to accept cholesterol from cells and to promote reverse cholesterol transport (RCT) represents the best characterized antiatherogenic function of HDL. Studies carrying out in animal models have unraveled the multiple mechanisms by which these lipoproteins drive cholesterol efflux from macrophages and cholesterol uptake to the liver. Moreover, the influence of HDL composition and the role of lipid transporters has been clarified by using suitable transgenic models or through experimental design employing pharmacological or nutritional interventions. Cholesterol efflux capacity (CEC), an *in vitro* assay developed to offer a measure of the first step of RCT, has been shown to associate with cardiovascular risk in several human cohorts, supporting the atheroprotective role of RCT in humans as well. However, negative data in other cohorts have raised concerns on the validity of this biomarker. In this review we will present the most relevant data documenting the role of HDL in RCT, as assessed in classical or innovative methodological approaches.

1. Introduction

Cholesterol homeostasis is critical for cell survival. Cellular mechanisms involved in its regulation include cholesterol uptake, synthesis, storage and efflux. All these mechanisms are responsible for maintaining the intracellular content of free cholesterol within a limited range. In particular, the ability to efflux excess cholesterol into extracellular acceptors, such high density lipoproteins (HDL), has been shown to be key in regulating processes such as inflammation [1], immune response [2] and insulin secretion [3,4].

In the context of atherosclerosis, the ability to efflux cholesterol from the lipid-laden macrophages in the arterial wall to HDL is considered the first and limiting step of a process known as reverse cholesterol transport (RCT), i.e. the transport of excess peripheral cholesterol to the liver to be eventually excreted. Macrophage RCT is considered one of the main atheroprotective functions of HDL [5].

In this review we will provide an overview of studies published in animal models and in humans demonstrating the role of HDL in promoting RCT and their correlation with cardiovascular disease (CVD).

2. Assessment of RCT in animals

The availability of animal models to study RCT represents an experimental advantage, allowing to analyze the role of HDL in individual steps of the process. Since the seminal definition of RCT by Glomset in 1968, an impressively large number of studies have been performed *in vivo*, in the attempt to provide insights into the mechanisms and regulation of this process. Whereas the works carried out in early times were primarily focused on the evaluation of centripetal flux of cholesterol from the entire extrahepatic system [6–8], in more recent years the development of a radioisotope-based assay have allowed to specifically measure the flux of macrophage-derived cholesterol to the liver to be ultimately excreted [9]. By tracing the most atherogenic pool of cholesterol along the RCT pathway, this method has provided multiple insights into this HDL function. Notably, while

most animal models have shown that a direct correlation between HDL-cholesterol (HDL-C) levels and RCT resulted in the protection towards atherosclerosis, the paradoxical case of scavenger receptor class B type I (SR-BI) KO mice, characterized by extremely elevated levels of HDL-C, the propensity to develop massive atherosclerosis when crossed with atherosclerosis-prone models [10–12], and reduced RCT [13], helped fueling the paradigm shift towards the evaluation of HDL functions, instead of HDL-C mass [14].

Whereas the role of the entire RCT pathway in atheroprotection is well established [14], the actual relevance of single steps of this process is still debated. Some studies highlight the importance of the final step, i.e. cholesterol and bile acid excretion into the feces, and interpret the extent of this elimination as a measure of the whole RCT process [15,16]. Although the regulation of this step is mainly dependent on the activity of ATP Binding Cassette G5 (ABCG5) and ABCG8 [17], the composition of HDL is also likely to play a key role [18]. Other studies focus on the first step, i.e. cholesterol efflux, as the most important one, given its role in influencing the pharmacokinetics (rate-limiting step) of the entire process and playing a pivotal, antiatherogenic role when the cholesterol-donor cells are macrophages [19,20]. It is, however, important to underline that an increase in tracer amount in circulation does not necessarily depend to improved cholesterol efflux, as it could be also due to an impaired hepatic uptake. Moreover, sometime the increased appearance of cholesterol in plasma does not translate in an increased fecal excretion, making the interpretation of the extent of whole RCT difficult.

In this debate, the actual properties of HDL in modulating RCT may not be easily attributable. Application of innovative methods to measure RCT in humans and animals, as discussed below, may contribute to provide insights in this challenging matter.

2.1 HDL-mediated cholesterol efflux from macrophages

The first step of RCT, i.e. the capacity of HDL to promote the release of excess cholesterol from cells, especially macrophages of the arterial wall, represents the best characterized, antiatherogenic

activity of HDL. It can be evaluated using an *in vitro* assay employing serum (or plasma) from animals as source of cholesterol acceptors incubated in the presence of cultured cells, usually macrophage cell lines. The use of cells specifically expressing lipid transporters, as well as parallel characterization of HDL composition, may provide a comprehensive characterization of the lipoprotein activity (Figure 1).

Figure 1
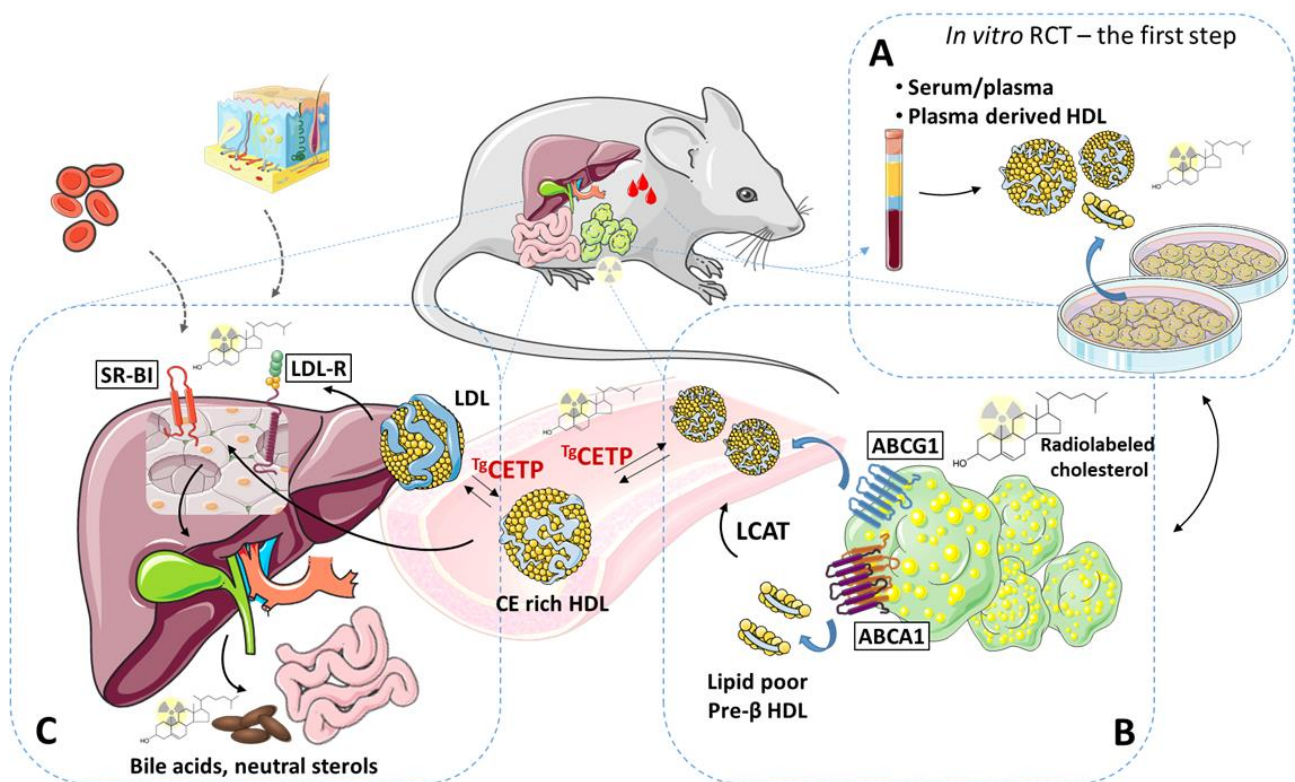
Experimental models of RCT in rodents. The standardization of methodologies allowing the cholesterol flux quantitation between cells and plasma components (e.g. HDL, apoA-I, etc.) dramatically changed the understanding of the RCT process. *A - In vitro* assays, based on cultured cells labeled with ³H-cholesterol help to characterize the first step of RCT, the cell cholesterol efflux towards extracellular acceptors. Whole mouse serum/plasma or purified lipoprotein fractions can act as physiological acceptors in such an experimental setting. *B, C – In vivo* assays contemplate the injection of ³H-cholesterol radiolabeled "foam cells" into the peritoneum of recipient mice and the assessing of macrophage-to-feces kinetic of the radiotracer. The injected cells exchange ³H-cholesterol through the efflux pathways (*B*). On the cell membrane, ABCA1 and ABCG1 transporters actively pump cholesterol towards lipid-poor or mature HDL particles, respectively. LCAT enzyme catalyzes the esterification of free cholesterol and the cholesterol ester (CE) enrichment of the HDL, that become larger and spherical. The vascular network (*center*) allows the transfer of the radiotracer throughout the mouse body along with the contemporary maturation of HDL particles. Mice naturally lack CETP, an enzyme catalyzing the transfer of CE from HDL to apoB-containing lipoproteins, such as LDL, in exchange with triglycerides. However, transgenic mice expressing human CETP (marked in red) have been

generated and helped in further clarifying the role of this enzyme in HDL metabolism (see the main text for details). HDL and LDL deliver cholesterol to the hepatocytes through the specific receptors SR-BI and LDL-R, respectively (*C*). Liver enzymes convert ^{3}H-cholesterol to bile acids (or neutral sterols) that are finally excreted into the feces. Red blood cells and skin actively contributes to cholesterol trafficking in the body.

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; LCAT, lecithin-cholesterol acyltransferase; TgCETP, transgenic cholesterol ester transfer protein; LDL-R, low density lipoprotein receptor; SR-BI, scavenger receptor-BI.

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More frequently, the capacity of HDL to accept cholesterol released from macrophages is evaluated *in vivo* as part of the RCT process, using a method that have been developed by Rothblat and Rader's labs [9]. This method assesses macrophage-specific RCT, injecting radiolabeled, cholesterol enriched macrophages into the peritoneal cavity of recipient mice (Figure 1). Cholesterol efflux occurs onto HDL and apolipoprotein A-I (apoA-I) diffused from the circulation into the peritoneum, where macrophages reside all along the experiment [9]. Once lipoproteins return in the blood compartment, the quantification of radioactivity in plasma can be carried out at different time points. It is, however, relevant to notice that, given the dynamic nature of the process, the amount of the tracer found in an intermediate compartment, such as the blood (or the liver), may not always be informative of the extent of the individual steps of the RCT.

Several, not yet completely defined, factors may contribute to HDL capacity to stimulate the first step of RCT *in vivo*. The influence of HDL composition has been investigated in several works, where the impact of genetic manipulation [13,21–32], pharmacological [19,33–38], nutraceutical or nutritional [39–43] interventions in mice were studied.

2.1.1 The influence of HDL size, lipid and protein composition

Size, lipid, and protein composition of HDL particles have been widely investigated. Small, lipid-poor HDL preferentially interact with ABCA1, whereas large, cholesterol-enriched subpopulations drive ABCG1- and passive diffusion-mediated processes [44]. These properties have a relevant

impact on macrophage RCT extent [45] and many *in vivo* studies integrate and deepen *in vitro* observations.

The ability of large, lipid-enriched HDL to promote macrophage RCT was demonstrated in mice treated with the liver X receptor (LXR) agonist T0901317. LXRs are nuclear receptors considered to be master regulators of cholesterol homeostasis in cells and the whole body [46]. Once activated by endogenous (e.g., oxysterols, desmosterol) or synthetic (e.g., T0901317, GW3965) agonists, LXRs stimulate the transcription of several genes important for the uptake (i.e, inducible degrader of the LDL receptor, IDOL), transport (i.e, apolipoprotein E, apoE) or efflux and disposal (i.e, ABCA1 and ABCG1) of cholesterol from macrophages [46]. An *in vitro-in vivo* integrated approach allowed to demonstrate that the increased mobilization of radioactive cholesterol along the RCT pathway compared to control animals was related, at least in part, to an increased capacity of HDL to promote passive diffusion efflux from cultured macrophages [47]. This study added a piece of information to the previously described capacity of LXR-mediated increase of HDL levels to promote RCT in mice [48]. Successively, LXR-mediated changes in HDL composition and function have been attributed to the receptors specifically expressed in the intestine, and not in the liver or macrophages [17].

Large HDL particles observed in human apoA-I transgenic mice treated with the non-nucleoside reverse transcription inhibitor efavirenz were associated with an increased ABCA1-independent efflux *in vitro* [49]. The increased HDL-C levels observed in this animal model was associated with an increased apoA-I production rate and an increase in *in vivo* macrophage RCT [49].

The role of small, lipid-poor prebeta-HDL in promoting macrophage RCT has been well established [23,34,50,51]. The first evidence was provided in a work employing the apoA-I mimetic peptide D4F. The oral ingestion by apoE null mice rapidly stimulated the formation of apoA-I enriched particles with prebeta mobility, increased efflux capacity from macrophages and increased macrophage RCT [34]. Consistently, the promoting activity of macrophage RCT by the cholesteryl

ester transfer protein (CETP) inhibitor dalcetrapib [52] and by rosuvastatin [53] has been attributed to the increased formation of prebeta-HDL. The impact of dynamic changes of HDL metabolism on macrophage RCT has been demonstrated in a recent study where a novel activator of lecithin cholesterol acyl transferase (LCAT) was administered to mice lacking the low density lipoprotein receptor (LDLR) or to cynomolgus monkeys. This compound produced an alteration in the turnover of prebeta-HDL, resulting in an increase of radioactive cholesterol appearance in plasma and feces of mice. As the authors suggested, the cholesteryl ester-enriched particles produced by the activation of LCAT efficiently promoted cholesterol efflux from macrophages and delivered cholesterol to the liver, without undergoing further increase in their size. The hepatic selective uptake of cholesterol from HDL caused the release of apoA-I, that in turn stimulated the RCT [54].

The quality and quantity of proteins carried into HDL particles is a critical determinant of size and efflux capacity. A recent, comprehensive work on isolated HDL from five inbred strains of mice assessed the contribution of individual proteins in regulating HDL efflux capacity. This study revealed that the content of apoA-I, apoC-III, apoD and phospholipid transfer protein (PLTP) strongly and positively correlated with HDL efflux capacity to promote cholesterol release from ABCA1-expressing cells, whereas the presence of apoE resulted in the opposite effect [55]. Consistently, it has been shown that HDL lacking apoE are smaller, preferentially prebeta-sized and efficiently promote cholesterol efflux from cultured macrophages as well as efficiently remove cholesterol through the RCT pathway [23].

The relevance of apoA-I in mediating HDL function in *in vivo* RCT has been directly demonstrated in studies where apoA-I overexpression significantly increased the amount of radioactive cholesterol mobilized from macrophages to plasma, liver and feces [9,56]. Conversely, the deletion of apoA-I (on a background of LDLR/apobec mice) caused the rearrangement of HDL towards larger particles with a reduced capacity to promote cell cholesterol efflux via ABCA1 and the impairment of radioactive cholesterol mobilization from macrophages to plasma and feces [30].

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Intriguingly, the expression of apoA-I$_{Milano}$, the mutated form of apoA-I whose expression causes severe hypoalphalipoproteinemia but low prevalence of atherosclerosis in carriers [57], did not ameliorate macrophage-to feces RCT in mice compared to animals expressing wild type apoA-I [58]. In the same study, the authors demonstrated that sera from both groups had similar cholesterol efflux capacity via ABCA1, whereas LCAT cholesterol esterification rate was impaired in plasma from mice carrying apoA-I$_{Milano}$.

Among other HDL associated apoproteins, apoA-II overexpression in mice, despite causing the decrease of HDL plasma levels, resulted in increased radioactive cholesterol mobilization from macrophages to liver and feces, without affecting radioactive content in plasma at the selected time points [31].

ApoA-IV overexpression in several mice models caused an increase in de novo biogenesis of discrete HDL particles, characterized by alpha mobility and spherical structure and an increase in cholesterol efflux *in vitro* [59]. The implications of this effect on RCT and atheroprotection are not yet established.

Interestingly, the enzyme paraoxonase, usually associated with the antioxidant activity of HDL [60], may represent a critical factor also for efflux capacity. In fact, its overexpression in mice, despite not altering circulating levels, produced HDL with an ameliorated efflux capacity towards cultured macrophages and resulted in the promotion of macrophage-to feces RCT [28].

Efflux capacity of either alpha and prebeta-HDL is largely influenced by the lipid composition of the particles, as well demonstrated in the work by Niisuke [44]. The reduction of phospholipids and cholesteryl ester content, as well as the increase of protein and triglycerides observed in wild type mice treated with tamoxifen blunted the efflux capacity from cultured cells and resulted in impaired *in vivo* macrophage RCT [61]. Interestingly, treatment with raloxifen, another estrogen receptor inhibitor, caused a less dramatic change in HDL composition and no significant changes in *in vivo* RCT [61].

2.1.2 The role of lipid transfer proteins

The influence of PLTP on HDL function in RCT is somewhat controversial. Whereas a positive correlation between this enzyme and HDL efflux capacity was demonstrated in a study testing *in vitro* sera from different mouse strains [55], the in vivo relevance of this observation was challenged by two works, where the overexpression of human PLTP in mice resulted in impaired mobilization of radioactive cholesterol from macrophages to plasma and feces [29,32].

In human CETP expressing mice, an experimental model that better recapitulates human lipoprotein metabolism, macrophage RCT is more efficient than control animals. The observed increased fecal excretion of radiolabeled cholesterol is possibly due to the transfer of cholesteryl esters from HDL to apoB-containing lipoproteins and the removal of circulating cholesterol through the LDLR pathway [17,22].

2.1.3 The role of ABC transporters and SR-BI

The role of hepatic expression of ABCA1 and SR-BI in regulating plasma concentration of HDL is well established [62,63]. Many *in vivo* studies provided further insights on the impact of these receptors in HDL function and RCT.

In a seminal work, Calpe-Berdiel and colleagues demonstrated that ABCA1-deficient mice have impaired macrophage RCT. The reduced appearance of radioactive cholesterol in plasma is thought to be driven by both the reduction of circulating HDL level, typical of ABCA1 lacking mice [64], and the less efficient interaction between these lipoproteins and ABCA1 deficient macrophages [65]. Conversely, the stabilization of ABCA1 protein by the addition of the small molecule IMM-H007 in the diet of wild type and apoE deficient mice stimulated radioactive cholesterol mobilization from macrophages to plasma, liver, bile and feces. This effect was related to the raise of HDL circulating levels and the improvement of their efflux capacity [66].

The specific role of hepatic ABCA1 in regulating HDL-driven RCT has been revealed in an elegant work by Bi and coworkers. Mice selectively deficient in the receptor expression of the transporter in the liver presented blunted levels of HDL, but surprisingly normal RCT. As postulated, the small pool of particles is sufficiently efficient in promoting cholesterol efflux to preserve the normal extent of the whole RCT. Intriguingly, the expected increase of small prebeta HDL, was not apparent, possibly related to the transient nature of these particles, that rapidly undergo catabolism and disappearance from the circulation [25]. Anyway, this study further strengthens the importance of the function rather than the quantity of HDL in its antiatherogenic activities.

The role of hepatic SR-BI in regulating lipoprotein levels is equally relevant. Its role in macrophage RCT is a well example of the possible discrepancy between HDL levels and function. Whereas the deletion of the hepatic expression of the scavenger receptor results in abnormal amount of circulating HDL, the impact on the overall process is the reduction of cholesterol mobilization, possibly related to dysfunctional HDL both in accepting cholesterol from macrophages and delivering cholesterol to the liver. Conversely, the overexpression of hepatic SR-BI, despite the low level of HDL, produced remnant particles and/or lipid free apoA-I that efficiently stimulate cholesterol efflux and release cholesterol to the liver [13].

Finally, the role of ABCA1, ABCG1 and SR-BI expression in macrophages on RCT was evaluated in a comprehensive study by Wang and colleagues. The results showed that ABCA1 and ABCG1, but not SR-BI, promote macrophage RCT *in vivo* and are additive in their effects [67]. Interestingly, in all the experimental models used in this study, the trend of radioactive cholesterol amount in plasma is consistent with that in the feces, suggesting that the first step is rate limiting of the entire process.

Factors that affect the ABC transporters and/or SR-BI expression have also been shown to affect HDL capacity to promote cholesterol efflux from cultured macrophages, as well as *in vivo* macrophage RCT. As mentioned above, LXR agonists are associated with increased *in vitro*

cholesterol efflux [47] and *in vivo* RCT [47,48]. Similarly, the activation of AMP-activated protein kinase (AMPK), a key regulator of lipid metabolism [68], has been shown to increase RCT *in vivo* and *in vitro* by increasing the expression of ABCA1 and ABCG1 in macrophages and SR-BI in the liver [69]. Treatment of apoE null mice under high fat diet with 4 different AMPK activators produced the increase of cholesterol mobilization from macrophages to plasma, liver and feces, in the absence of alteration of circulating levels. Concurrently, apoB-depleted sera from these animals revealed efficient cholesterol removal from ABCA1-expressing macrophages in culture, indicating that the promotion of the first step of RCT is sufficient to stimulate the whole process [69].

2.1.4 The impact of nutritional interventions

Animal models undergoing nutritional interventions represent a useful model to investigate how dietary factors may affect HDL activity in macrophage RCT [70,71]. Improved cholesterol efflux and macrophage RCT *in vivo* has been observed in mice treated with polyphenol-enriched olive oil, suggesting that these micronutrients are critical in regulating this function [40]. HDL-C levels, *in vitro* ABCA1-independent cholesterol efflux capacity and macrophage-to-plasma RCT *in vivo* were increased in mice fed high fat diets enriched of either saturated or monounsaturated fats, as compared with a low-fat diet. However, later steps of RCT, namely liver-to-feces RCT, were increased only in animals fed the high fat diet enriched in monounsaturated fats [18]. The authors proposed that the proinflammatory phenotype of HDL proteome in mice fed a saturated fat-enriched diet could be responsible for the impairment in cholesterol transport from liver to the feces compared to mice undergoing a low-fat diet. Interestingly, the authors speculated that the accumulation of radioactive cholesterol in adipose tissue observed in the same animals, may counterbalance the impaired fecal excretion, and neutralize CVD risk.

2.2 Hepatic cholesterol uptake

The release of cholesterol from HDL to the liver is one of the last steps of RCT and can occur through three main mechanisms. The first is the interaction of the particles with hepatic SR-BI,

leading to the selective uptake of free cholesterol and cholesteryl esters and the consequent generation of de-lipidated HDL. These particles are efficient cholesterol acceptors, thus promoting cholesterol efflux from peripheral cells and further stimulating RCT [72]. This mechanism is the main pathway in most mouse models. The second is mediated by CETP and relies on the transfer of cholesteryl esters from HDL to apoB-containing lipoproteins, that in turn are picked up by the liver through the LDLR. This is the main mechanism in humans [73], but not in mice that do not naturally express CETP. In mice engineered to express CETP, RCT was improved, despite the expected reduction of HDL levels, as demonstrated by the increased excretion of fecal cholesterol [22], suggesting that the efficient delivery of cholesterol to the liver via CETP is critical for the rate of the whole process. The overexpression of CETP in mice provides a suitable model to investigate the role of HDL remodeling in influencing RCT through an SR-BI-independent HDL clearance. In SR-BI deficient mice, as well as in liver-specific SR-BI KO mice, CETP expression normalized the plasma HDL-C levels. However, the plasma free cholesterol:total cholesterol ratio, which is elevated in SR-BI deficient animals, was not corrected by the transgenic CETP. Moreover, the impaired macrophage to feces RCT observed in SR-BI deficient mice remained unaffected by CETP expression [74].

Finally, a process of hepatic cholesterol uptake, independent of SR-BI, has been proposed as the mechanism accounting for the increased mobilization of radioactive cholesterol in apoA-II-enriched HDL [31], although not yet well characterized.

During *in vivo* RCT experiments, the capacity of HDL to deliver cholesterol to the liver can be quantified as the hepatic content of radioactive cholesterol, with the caveat that the evaluation of a single time point (that at sacrifice) may not reflect the efficiency of this specific step during the length of the experiment. This is probably the reason why many studies in which animals are sacrificed 48h after the injection of radioactive cholesterol-enriched macrophages displayed similar accumulation of tracer in the liver as the control groups, despite differences in fecal excretion.

HDL size and composition may affect not only its efflux capacity, but also its ability to release of cholesterol to the liver. Overexpression of apoE in mice resulted in a raised increase in hepatic radioactive cholesterol uptake [21]. Interestingly, it did not result in an increase of fecal sterol excretion, compared to wild type mice, due to a potential competition of cholesterol availability between excretion into the bile and re-secretion in the plasma via ABCA1. This suggests that enhancement of cholesterol uptake by the liver does not necessarily translate in increased last step of RCT.

Consistent with this hypothesis are the results observed by Bashore and colleagues in liver-specific ABCA1 null mice [75]. Despite the smaller pool of HDL circulating in plasma, radiolabeled cholesterol uptake by the liver and fecal excretion were increased in these mice as compared to control mice, while re-secretion of the radiolabeled cholesterol on HDL was significantly reduced, suggesting that the up-taken cholesterol was preferentially targeted for excretion into the feces, instead of secretion into plasma, thus stimulating the overall RCT [75].

A comprehensive view on the implications of HDL size/function in cholesterol hepatic uptake and RCT efficiency has been provided by a recent study in mice overexpressing endothelial lipase. These animals presented lower levels of circulating HDL that were overall smaller and with a lower amount of lipids as compared with control mice [76]. Despite a lower amount of counts in circulation, there was no difference in fecal counts, due to the enhanced ability of cholesterol uptake in the liver via SR-BI [77].

HDL turnover studies employ another informative technique to evaluate the last steps of RCT, namely cholesterol liver uptake and fecal excretion. This method relies on radiolabeling HDL collected from donor animals or humans, their intravenous injection into recipient animals and the eventual quantification of HDL-derived radioactive cholesterol in the liver (either as free or esterified form) and feces (both in neutral sterol and bile acid fractions) [21,41,43,74]. Using this method Nishimoto and colleagues demonstrated that the enrichment of HDL in polyunsaturated

fatty acids in wild type mice fed with fish oil promoted macrophage RCT primarily through a stimulated elimination of HDL-derived cholesterol via liver and feces, and to a lesser extent for the increased capacity of serum to promote cholesterol efflux via ABCA1 [43].

2.3 Alternative pre-clinical models for the assessment of RCT

Although mouse models are extensively used to study RCT, they have some limitations that may reduce the translatability of the results to human physiopathology. One of the major lack is the absence of CETP in mice. In this context, additional value may be attributed to studies performed in more human-like models, such as hamsters. In this animals the assessment of macrophage to feces RCT can be performed as originally developed in mice [52,78]. Briand and colleagues demonstrated that dyslipidemia, despite inducing the increase of circulating HDL levels, impairs macrophage RCT. This effect is partially explained by the reduced capacity of these particles to promote cholesterol efflux, suggesting the appearance of dysfunctional HDL [79]. Similarly, metabolic syndrome, induced in hamsters fed a high fructose and high fat diet, showed altered macrophage RCT despite the increase in HDL levels. The observed increase in radioactive cholesterol in plasma, was possibly related to this abnormally increased levels of lipoproteins, rather than to an improved efflux process [39]. Hamsters represent a valuable model also for pharmacological/nutraceutical studies assessing the potential anti-atherosclerotic activity of novel compounds. As an example, Romain and colleagues tested the effect of a novel polyphenol-based dietary supplement on plasma lipoprotein profile of Golden Syrian hamsters, demonstrating both the remodeling of HDL towards smaller particles and increased capacity to promote ABCA1-mediated efflux. Despite not directly assessed, the reported reduction of atheroma development was probably driven by stimulation of macrophage RCT [50].

In recent years it has been demonstrated that HDL capacity to accumulate in lymphatic vessels is critical for their activity in the promotion of macrophage RCT [80]. In mice receiving local vasoactive stimuli able to enhance vascular permeability, recruitment of HDL at the peripheral

interstitial fluids resulted in the net removal of cholesterol from macrophages in the subcutaneous layer of the skin into the feces [81].

Finally, red blood cells have been proposed as cholesterol sink, able to contribute to the efficient RCT process to the feces [82]. Although the authors suggested a process at least in part independent of HDL, successively the relevance of these lipoproteins in exchanging cholesterol with red blood cells was demonstrated *in vitro* [83,84]. The actual *in vivo* implications of these observations are still uncertain and further studies are necessary to address this question.
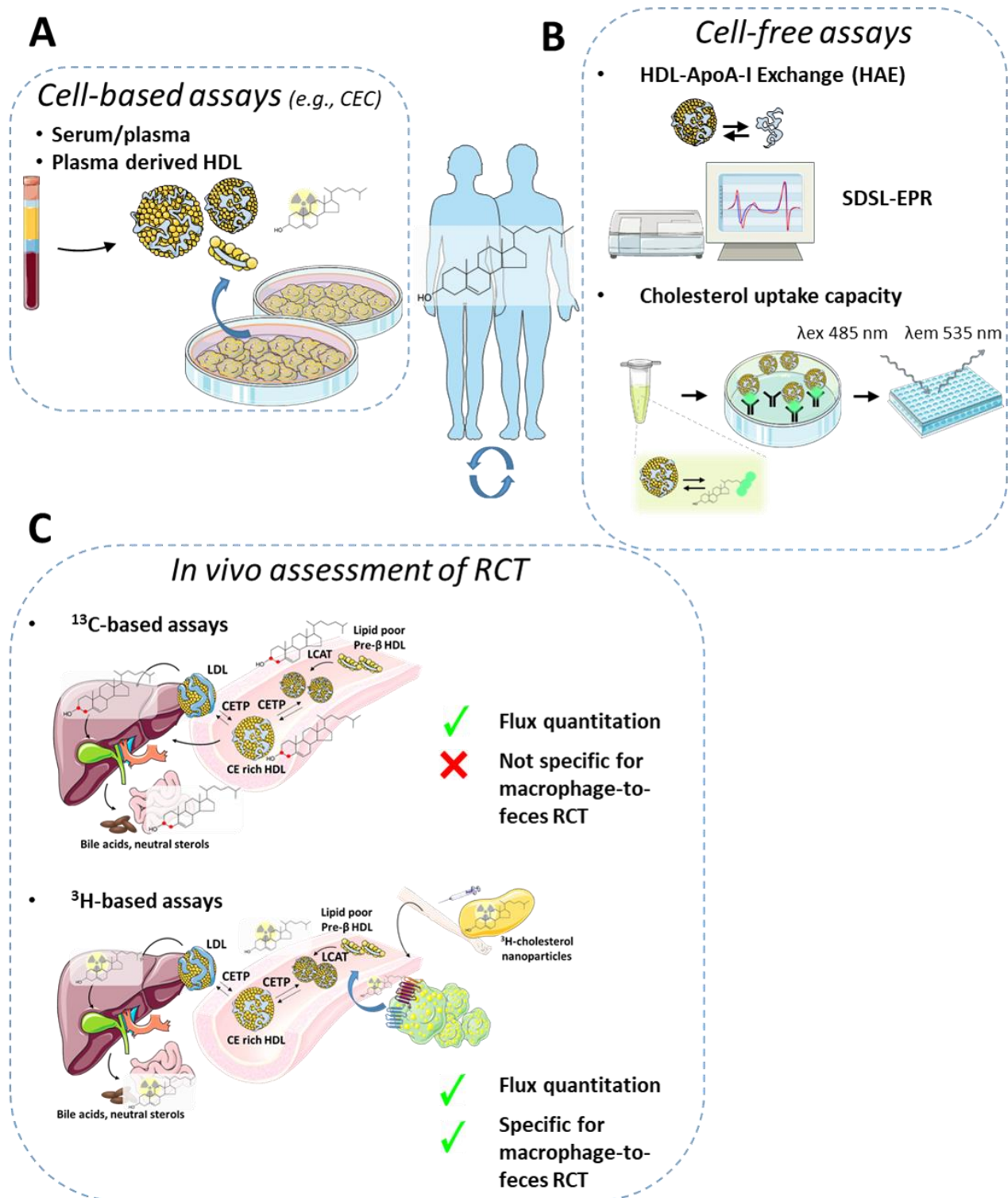
3. Assessment of RCT in humans

3.1 HDL-mediated cholesterol efflux

As the complexity of the RCT methods used in pre-clinical models makes it hard to translate them in humans, most of the attention to investigate this HDL function has been dedicated on developing an assay to assess the first step of RCT, namely the ability of HDL to accept cholesterol from macrophages. The ability to efflux cholesterol to HDL and the mechanisms underlying this process have been extensively characterized in the laboratories of Dr. Rothblat and Phillips. They were the first to investigate the mechanisms of cell cholesterol efflux in detail [85] and to demonstrate the role of HDL composition in influencing the capacity of these particles to exchange cholesterol with cells [86–88]. Later on, after the discoveries of the key role of lipid transporters present on the cell plasma membrane, they widely contributed to provide insights into the mechanisms of HDL interactions with ABCA1 [76,89–91], ABCG1 [92], and SR-BI [76,91,93,94], and to fully characterize the individual contribution of these mechanisms, including passive diffusion, in the regulation of cell cholesterol homeostasis [45,95]. The *in vitro* assessment of cholesterol efflux capacity (CEC) of human sera was originally proposed in a study demonstrating the relationship of efflux function with HDL content [96] and it was successively ameliorated using radioisotopes or fluorescent tags to trace the amount of labeled cholesterol transferred from donor cells to acceptors present in the media, usually apoB-depleted serum [97–99].

Based on these studies, the most used systematic approach to measure human HDL CEC is an *in vitro* method that assesses the ability of an HDL preparation obtained from human samples to promote cholesterol efflux in the presence of donor cells (Figure 2A). Several reports used this approach to investigate the cholesterol efflux capacity in individuals carrying pathogenic variants in genes encoding for key HDL proteins (see for example [100–103]). However, the interpretation of differences in CEC between carriers and controls is complicated by the presence of low HDL levels as well as other factors, such obesity or diabetes, known to affect HDL levels and function. Interestingly, early studies using an alternative approach (i.e. fibroblasts obtained from patients and incubated with apoA-I or a standard HDL preparation) had clearly demonstrated the decreased ability to efflux cholesterol of cells obtained from patients with ABCA1 mutations, but not from patients with other forms of familial hypoalphalipoproteinemia [104,105].

Figure 2

Experimental models of RCT in humans. The most characterized methods allowing the assessment of RCT in humans are shown. They contemplate cell-based (*A*) or cell-free (B) in vitro assays, and more complex methodologies for investigating RCT in vivo (*C*). *A* - Cholesterol efflux capacity (CEC) is an in vitro method that assesses the ability of a HDL preparations or apoB-depleted plasma to promote cholesterol efflux from cells (e.g., cholesterol normal/loaded macrophages or other cell models). *B* – Cell-free tests measuring either the

ability of apoA-I of exchange, using electron paramagnetic resonance spectroscopy, or cholesterol uptake capacity, using an anti-apoA-I antibody to capture HDL, coupled to spectrometry to detect the fluorescently labeled cholesterol incorporated into the lipoprotein particle. *C* - Two methods assessing cholesterol flux *in vivo* in humans: the first, using stable-isotope (^{13}C, marked as red dots) labeled cholesterol; the second using radiolabeled (^{3}H) cholesterol, which closely mimics the methods applied to murine models. The specific advantages and disadvantages of the two approaches are highlighted in the figure.

Abbreviations: CEC, cholesterol efflux capacity; HAE, HDL-apoA-I-Exchange; SDSL-EPR, site-directed spinlabel electron paramagnetic resonance; λex, excitation wavelength; λem, emission wavelength; LCAT, lecithin-cholesterol acyltransferase; CETP, cholesterol ester transfer protein.

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Results for this method varies depending on the type of acceptor and donor cells used in the assay [99,106] and it is not yet standardized for clinical use. However, an inverse association between CEC and either prevalence or incidence of CVD has been found in most studies utilizing apoB-depleted serum/plasma containing HDL as acceptors particles and macrophage cell lines as donor cells.

Following the first report of an inverse correlation between CEC and prevalent atherosclerotic cardiovascular diseases (ASCVD) independently of HDL and apoA-I levels [107], Rohatgi et al reported a similar finding for incident ASCVD in the Dallas Heart Study [108]. Several other studies have replicated these results in different populations for both prevalent and incident ASCVD [109–117] as well as mortality in ASCVD patients [118]. Interestingly, in the MESA cohort studied by Shea [116] and colleagues a strong inverse correlation was found with coronary artery disease events, but not with stroke, confirming what observed by Zhang et al. [111], suggesting that factors driving this association may differ depending on the vascular beds involved.

Although most studies using such assay have found an independent inverse relation with ASCVD, other studies have not. Notably, Li and colleagues [112] were the first to report discordant results using an assay similar to the one used by Khera et al. [107]. In that study, an inverse association with prevalent ASCVD was found in an outpatient cohort, while a paradoxical direct association was found with incident ASCVD in an older cohort, enriched with smokers [112]. Discordant

results were also found in a study that enrolled patients with the high inflammatory markers C reactive protein [119], in a cohort of patients with type 2 diabetes [120] and in patients with chronic kidney disease (CKD) [121–123]. A large longitudinal cohort that followed healthy men over 16 years found an inverse correlation between CEC and coronary heart disease (CHD) risk, but this association was completely attenuated once controlled for HDL-C [124]. It cannot be excluded that some discrepancies are due to methodological differences of the assays used in these studies. However, the paradoxical association of CEC with increased cardiovascular risk in patients with CKD and not in those with prevalent CVD, and the inverse correlation in the control group, was confirmed using the same method [121]. As most of these studies are in cohorts of patients with conditions known to significantly alter lipoprotein metabolism, it is possible that the inverse correlation between CEC and ASCVD is lost when the HDL composition is affected by the underlying condition. Both diabetes and CKD are characterized by elevated prebeta-particles and alteration of the HDL proteome [125,126]. Elevated prebeta-particles have been also found in several dyslipidemias, particularly in the presence of hypertriglyceridemia [127] and in ASCVD patients [128]. Although prebeta-particles in these patients are probably dysfunctional [129], the use of an *in vitro* assay that is particularly sensitive to ABCA1 pathway [97,130] may produce results that are not reflective of RCT. The possibility that the ability of CEC to predict ASCVD is lost in conditions associated with dysfunctional HDL is also suggested by the results observed in the JUPITER trial [119]. In this trial, enrolling subjects with elevated C-reactive protein levels, CEC was not predictive at baseline, but it became so after 1 year in statin treatment. Consistently, results of a meta-analysis in patients with rheumatoid arthritis suggested that treatments that improve highly inflammatory conditions may also restore HDL-mediated CEC [131].

Interestingly, in those studies where HDL particle number were also assessed, this parameter resulted a better biomarker than CEC to predict ASCVD events also in the context of high level of inflammation of CKD [119–121].

Finally, the predictive value of CEC may be lost in healthy populations in which longitudinal risk tracks very well with HDL-C levels [124] or in an elderly population as the one evaluated by Li and colleagues [112]. No inverse association between CEC and atherosclerotic burden was also found in a small cohort of healthy octogenarians [132]. Interestingly, CEC levels in this cohort of very old individuals were significantly higher than that found in a group of middle age controls.

3.1.1 HDL subspecies

The discrepant results reported in several cohorts raise the question if HDL CEC can be considered a reliable biomarker. Thus, the exploration of better suited biomarkers continues.

It is now appreciated that HDL are vehicles carrying many different proteins [133,134], as well as different lipids [135] and microRNAs [136], that confer HDL many other functions beyond those of cholesterol acceptor and lipid transporter. With this realization, the concept of HDL subspecies (as groups of HDL particles that are characterized by the presence of a specific protein cargo), as opposed to the concept of HDL subfractions (as a group of particles that have similar size but not necessarily a similar cargo), is gaining momentum [137].

Furtado et al. have identified distinct proteomic signatures in 16 HDL subspecies, of which only some contain proteins involved in lipid metabolism, while others carry proteins associated with other HDL functions, such as immunity, antioxidation, or anti-inflammation [138]. The same group has identified HDL subspecies containing apoE and apoC-I as atheroprotective [139], while those containing apoC-III as associated with an higher risk of incident CHD [140]. Small HDL particles isolated from patients with type 2 diabetes have been shown to exhibit defective ability to accept ABCA1-mediated effluxed cholesterol as compared to those of controls, and to have altered content of triglycerides, apoC-II and SERPINA1. Interestingly, apoC-II and SERPINA1 levels were found to correlate with ABCA1-mediated CEC, and supplementing SERPINA1 in to the small HDL particles restore this efflux pathway [141]. Hopefully, further characterization of HDL subspecies

will result in the identification of biomarkers strongly and reliably associated with RCT, that would also be easily implementable in the clinical setting.

3.1.2 Cell-free methods as a measure of HDL-mediated cholesterol efflux

CEC assay is not easily adaptable in the clinical setting due to the use to cell cultures and radiolabeled cholesterol. Novel cell-free approaches have been proposed to circumvent this problem (Figure 2B). A test uses electron paramagnetic resonance spectroscopy to measure the ability of apoA-I to exchange, as a marker of HDL ability to remodel [142]. HDL-apoA-I exchange has been shown to correlate with total and ABCA1-mediated efflux, accounting for approximately 25% of the variance [143], to inversely correlate with the presence of ASCVD [142] and to be reduced in patients with diabetes [144]. A test evaluating cholesterol uptake capacity using a fluorescence signal to identify labeled cholesterol incorporated into HDL captured by an anti-apoA-I antibody has been shown to be inversely correlated with recurrence of coronary lesion after revascularization in patients with optimal control of LDL-cholesterol levels [145] and with lipid-rich plaque burden and macrophage content measured using optical coherence tomography [146]. Although these cell-free tests may represent a more promising approach to scale cholesterol efflux assays in the clinical setting, they remain to be fully tested in larger cohorts, and to evaluate if they can be better predictors in patients with diabetes and other conditions where CEC does not provide reliable results.

3.2 Hepatic cholesterol uptake

As mentioned before, the ability to deliver excess cholesterol to the liver is an important step of RCT. *In vitro* assays using a hepatic cell line, such HepG2 cells, incubated with serum or plasma obtained from patients or heathy volunteers are being used to assess the ability of HDL to deliver cholesterol to the liver, as one of the final steps of RCT [147,148]. Although plausible, this approach would need to be extensively validated before it could be used to extrapolate conclusions that are relevant to the human physiopathology. This is particularly important as, contrary to mouse

models that do not contain CETP, HDL kinetics studies in humans strongly suggest that the majority of cholesterol carried by HDL is delivered to the liver via the CETP/LDLR axes [73].

3.3 Assessment of whole body RCT in humans

One of the major limitations of CEC is that it is an *in vitro* assay that does not necessarily reflect what happens *in vivo*. The strongest evidence of the atheroprotective effect of HDL, and specifically of the central role of RCT, comes from *in vivo* studies in animal models, as detailed in the previous sections. Similarly, *in vivo* metabolic tracer studies have been instrumental in advancing our understanding of HDL metabolism in health and disease in humans [73]. Thus, investigating the metabolic fate of HDL *in vivo* can provide a better assessment of RCT in humans. Recent studies performed using endogenous labeling of proteins with stable isotopes suggest that, contrary of the classical HDL model in which prebeta, nascent HDL particles are secreted by the liver and expand in size in circulation, HDL of different sizes are secreted in circulation at the same time, and that only a fraction of them can change size, probably as a consequence of fusion and remodeling [149]. Of note, the secretion of multiple size HDL is also supported by data obtained *in vitro* [150,151]. These studies were expanded by Morton et al. [152] that found that HDL subspecies containing apoE actively expand in size in circulation and then are quickly cleared, consistent with the RCT function of HDL. Interestingly, the co-presence of apoC-III inhibits the catabolism of these HDL particles [152].

Although labeling of proteins carried by HDL may offer an indirect assessment of RCT *in vivo* by studying the flux of these proteins between HDL particles of different size, it does not offer a direct measurement of the transport of cholesterol. In recent years, two methods have been developed to assess cholesterol flux *in vivo* in humans (Figure 2C). The first, developed by Turner et al. [153], measures the systemic whole-body flux of cholesterol from the periphery to the feces, through three primary components of RCT: tissue cholesterol efflux, esterification of free cholesterol ~~FC~~ in plasma and fecal excretion of plasma-derived free cholesterol. Using this method, Holleboom et al.

[154] demonstrated that tissue cholesterol efflux was significantly reduced in patients with low HDL-C levels due to mutations in APOA1, supporting the role of HDL in the efflux of cholesterol from peripheral tissues, but no changes in fecal sterol secretion were observed. This method was also used to assess the effect of ezetimibe, a lipid-lowering drug that inhibits the absorption in intestinal cholesterol. Treatment with ezetimibe significantly reduced LDL-C levels, without changes in HDL-C levels, and significantly increased the secretion of plasma-derived cholesterol as neutral sterols [155]. Although these results are providing important insights into cholesterol metabolism, this method is not macrophages-specific and cannot provide the necessary insight in the context of atherosclerosis. The second method, based on the studies performed by Schwartz and colleagues [73], uses labeled cholesterol nanoparticles to selectively follow macrophage-specific RCT. It was extensively validated in animal models [156] , and can provide measure of key RCT steps, such as cholesterol efflux from macrophages, LCAT-dependent cholesterol esterification and CETP-dependent cholesterol transfer to LDL through multicompartmental modeling. The results suggested that it could offer a valid approach to measure rates of macrophage RCT *in vivo* in humans [156], but further studies are needed.

In vivo studies have also been instrumental to highlight the role of the lymphatic system in the metabolism of lipoproteins, and HDL in particular. The topic has been recently extensively reviewed [157,158]. Briefly, in humans lipoprotein concentration and composition in lymph is different from plasma [159]. Lymph is enriched of HDL, whose size distribution is broader than in plasma, spanning from very large particle enriched in unesterified cholesterol to prebeta-particles enriched in phospholipids [160]. Infusion of reconstituted HDL in healthy volunteers resulted in an increase in prebeta-particles and cholesterol content in lipoproteins in the afferent lymphatic fluid as compared with plasma [161]. Further studies suggest that, while in plasma the role of the more abundant alpha-HDL particles is to carry cholesterol ester to hepatocytes, in the interstitial fluids the milieu facilitates the conversion of alpha-HDL to prebeta-HDL, that in turn are better acceptors

of free cholesterol effluxed from peripheral tissues [162]. These findings are supported by studies in animal models, that demonstrated the critical role of lymphatic vasculature in RCT [80,163] and atherosclerosis [164]. They also highlight the need to better understand the mechanisms regulating HDL metabolism in the interstitial fluid and its transport through the lymphatic vasculature, in the attempt to develop better strategies to enhance RCT.

4. Therapeutic strategies to enhance RCT

Developing therapeutic approaches that enhance RCT remains an important goal. Most approaches aim at increasing the production or the availability of functional circulating HDL, and include infusion of reconstituted HDL, drugs increasing apoA-I production (such as new generations of PPARα agonists and bromodomain inhibitors), drugs targeting ABCA1 and cholesterol efflux (such as LXR agonists or miRNA), and others [165,166]. However, translating promising findings from animal models to humans has proven challenging. The two approaches that have been more extensively tested in humans are reconstituted HDL infusions and LXR agonists.

Studies in animal models have strongly supported the atheroprotective effect of infusion of reconstituted HDL administration (See Stoekenbroek et al. for an extensive review [167]). Initial results in small cohorts of humans have provided encouraging results, but inconclusive at best [168–171]. Consequently, most programs have been abandoned. CSL-112 is the only reconstituted HDL still in development. Histological changes in atherosclerotic plaques were observed in a small study using a first-generation formulation, CSL-111 [172]. Subsequent studies conducted with CSL-112 have shown safety, as well as a significant increase in prebeta-particles and CEC [173–176]. A phase 3 trial to investigate effect of CSL-112 infusion on cardiovascular outcomes is still ongoing (NCT03473223) [177] and results eagerly awaited. Promising, preliminary results have been also observed in high-risk patients with infusion of autologous prebeta-particles obtained by an HDL delipidation process [178,179], but they need to be confirmed in larger, placebo-controls trials.

Numerous pre-clinical studies have also supported the use of LXR agonists. However, in addition to its role in the activation of key genes regulating RCT in the macrophages, LXR also regulates genes associated with hepatic lipogenesis and very low-density lipoproteins secretion, resulting in hepatic steatosis and hypertriglyceridemia. Several LXR agonists that have advanced in clinical trials have not been so far successful [180,181]. Interestingly, the administration of an LXR agonist (T0901317) encapsulated in synthetic HDL seems able to specifically target the macrophages and bypass signs of liver toxicity [182,183]. The optimized formulation showed increased *in vitro* CEC and decreased atherosclerosis development in apoE-deficient mice as compared to T0901317 alone or synthetic HDL [182]. More studies are needed to determine the translatability of these promising results in humans.

Recently, desmocollin 1 (DSC1) has been indicated as an attractive pharmacological target for HDL biogenesis. In specific plasma membrane micro-domains, DSC1 can act as a molecular "trap" for apoA-I, subtracting it to the interaction with ABCA1 and preventing HDL biogenesis [184]. Thus, inhibiting the apoA-I/DSC1 interaction by downregulating DSC1 expression through RNA interfering, genomic manipulation (e.g., CRISPR/Cas9 approach) or by using specific DSC1-blocking antibodies may restore the apoA-I/ABCA1 interplay and increase cellular cholesterol efflux. However, this new therapeutic approach has only been evaluated *in vitro* so far and new pre-clinical and clinical studies are expected to demonstrate its efficacy also in animal models and humans, respectively.

Finally, although the antiatherosclerotic effect of LCAT is still being debated, several drugs targeting LCAT are at different stages of development [185]. A novel LCAT activator, DS-8190a, was able to stimulate RCT in mice overexpressing human LCAT and in cynomolgus monkeys [186]. In a phase 2a study, a recombinant human LCAT formulation showed good safety and tolerability as well as increased HDL-C levels [187]. A Phase 2b on the same molecule is currently ongoing (NCT03578809).

5. Conclusion and future directions

Macrophage cholesterol efflux and RCT transport pathway are among the most studied and characterized functions of HDL. Animal studies have undoubtedly highlighted the importance of these HDL functions for atheroprotection and are instrumental to identify potential therapeutic targets. CEC in humans has been shown to be associated with both incident and prevalent cardiovascular events in several populations, but not in other, raising concerns in regard to the generalizability of the use of this biomarker. Methods to assess RCT *in vivo* in humans have been developed, that could be used during the development of novel therapeutic strategies. However, more research needs to be conducted to better characterize HDL subspecies and identify easy to test biomarkers for clinical application.

CRediT author statement

Ilaria Zanotti: Conceptualization; Writing - original draft; Writing - review & editing; **Francesco Potì**: Visualization; Writing - review & editing; **Marina Cuchel**: Conceptualization; Writing - original draft; Writing - review & editing.

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