Rethinking Reverse Cholesterol Transport and Dysfunctional High Density Lipoproteins

Baiba K. Gillard, PhD[a,b,*], **Corina Rosales, PhD**[a,b,*], **Bingqing Xu, MD**[c], **Antonio M. Gotto Jr., MD, DPhil**[a,b], and **Henry J. Pownall, PhD**[a,b]

[a]Center for Bioenergetics, Houston Methodist Research Institute, 6670 Bertner Avenue, Houston TX 77030

[b]Weill Cornell Medicine, 1305 York Avenue, New York, NY, 10065 USA

[c]Department of Cardiovascular Medicine, Xiangya Hospital, Central South University, Changsha 410008, China

Abstract

Human plasma high density lipoprotein-cholesterol (HDL-C) concentrations are a negative risk factor for atherosclerosis-linked cardiovascular disease (ACVD). Pharmacological attempts to reduce ACVD by increasing plasma HDL-C have been disappointing so that recent research has shifted from HDL-quantity to HDL-quality, i.e., functional vs. dysfunctional HDL. HDL has varying degrees of dysfunction reflected in impaired reverse cholesterol transport (RCT). In the context of atheroprotection, RCT occurs by two mechanisms: One is the well-known trans-hepatic pathway comprising macrophage free cholesterol (FC) efflux, which produces early forms of FC-rich nascent (n)HDL. Lecithin:cholesterol acyltransferase converts HDL-FC to HDL-CE while converting nHDL from a disc to a mature spherical HDL, which transfers its CE to the hepatic HDL receptor, scavenger receptor B1 (SR-B1) for uptake, conversion to bile salts, or transfer to the intestine for excretion. Although widely cited, current evidence suggests that this is a minor pathway and that most HDL- and nHDL-FC rapidly transfer directly to the liver independent of LCAT activity. A small fraction of plasma HDL-FC enters the trans-intestinal efflux pathway comprising direct FC transfer to the intestine. SR-B1$^{-/-}$ mice, which have impaired trans-hepatic FC transport, are characterized by high plasma levels of a dysfunctional FC-rich HDL that increases plasma FC bioavailability in a way that produces whole body hypercholesterolemia and multiple pathologies. The design of future therapeutic strategies to improve RCT will have to be formulated in the context of these dual RCT mechanisms and the role of FC bioavailability.

Address for Correspondence: Henry J. Pownall, Houston Methodist Research Institute, Room R11-217, 6670 Bertner Avenue, Houston TX 77030. hjpownall@HoustonMethodist.org Tel: (713) 449-4537; FAX (713) 363-8936.
*Shared first authors.

Keywords

reverse cholesterol transport; HDL biogenesis; atherogenesis; ATP-binding cassette transporter A1; lipoprotein receptors; cholesterol bioavailability

Subject Codes

Lipids and cholesterol; metabolism; atherosclerosis; cell biology/structural biology

The Reverse Cholesterol Transport (RCT) Hypothesis

Atherosclerotic cardiovascular disease (ACVD), a major cause of mortality and morbidity in the United States,(3) is characterized by cholesterol accumulation within the subendothelial space of the arterial wall. Discovery of the reaction of lecithin:cholesterol acyltransferase (LCAT), which esterifies free cholesterol (FC), mainly on high density lipoprotein (HDL), provoked the hypothesis that LCAT is part of a broader cholesterol transport process that sequesters cholesterol as cholesteryl ester (CE) in HDL thereby preventing accumulation of cytotoxic FC levels in peripheral tissues, which cannot catabolize FC.(2,4,5) This process (Figure 1), reverse cholesterol transport (RCT), was hypothesized before the discovery of cellular cholesterol transporters and HDL receptors. In the first RCT step, interaction of apo AI with the cellular lipid transporter ATP-binding cassette subfamily A, member 1 (ABCA1),(6,7) forms nascent HDL (nHDL), which contains mainly apo AI, free cholesterol (FC), and phospholipid (PL);(8,9) though less studied, a related lipid transporter, ABCG1, mediates FC efflux to HDL.(10) In the context of atheroprotection, the first step occurs on macrophages within the subendothelial space of the arterial wall. The second step, nHDL esterification by plasma LCAT converts discoidal nHDL to a mature spherical HDL with a central CE core. In the final step—cholesterol disposal—hepatic scavenger receptor class B member 1 (SR-B1), selectively extracts HDL-lipids (11) for disposal in the bile by a nibbling mechanism in which HDL-apos are excluded from uptake; HDL is reduced to a remnant while releasing lipid-free apo AI.(12) Although cited to near-consensus,(13,14) this trans-hepatic RCT model has not been fully validated.

Spontaneous Lipid Transfer

Plasma lipids transfer among lipoproteins and cells by several mechanisms. Spontaneous transfer, which occurs without the aid of transfer proteins, occurs on a physiologically meaningful time scale for FC, PL, and non-esterified fatty acids (FA). Halftimes ($t_{1/2}$) for FC transfer from HDL_3 and low density lipoproteins (LDL) to lipoprotein and membrane surfaces are respectively 3 and 45 min.(15) FA transfer halftimes vary according to chain-length and unsaturation. Each methylene unit and double bond decrease and increase respectively the transfer $t_{1/2}$ by a factor of ~4.(16) The effects of chain length on FA transfer rates are profound. The $t_{1/2}$ for palmitic acid (16-carbons) transfer is ~3 ms; adding ten methylene units, which gives hexacosanoic acid, increases $t_{1/2}$ to 3 hours. Hexacosanoic acid is associated with adrenoleukodystrophy, an inherited disorder of FA metabolism in which hexacosanoic acid accumulates in tissues due to slow transfer to peroxisomes for

degradation.(17) PL transfer rates also vary similarly with acyl chain composition. The $t_{1/2}$ for PL and sterol transfer increases with increasing particle size, HDL < LDL < VLDL. (15,18) Given that they are a major component of nHDL,(1,8,9) the fate of nHDL-PL in the RCT pathway is determined in part by acyl chain composition and nHDL size.

Plasma HDL-Modifying Activities

HDL is modified by several plasma enzymes, transfer proteins, and cell surface receptors—cholesteryl ester transfer (CETP),(19) and phospholipid transfer proteins (PLTP),(20) LCAT, (21) endothelial(22) and hepatic lipases,(23) and hepatic SR-B1.(12) CETP transfers CE, triglyceride (TG) and PL among lipoproteins;(24) its most medically relevant activity is exchange of LDL- and HDL-CE for VLDL-TG, which in the context of hypertriglyceridemia gives rise to TG-rich LDL and HDL,(25) which are subsequently reduced to small, dense LDL and HDL_3.(25) The in vivo $t_{1/2}$ of CE transfer activity is on the order of 4–6 h(26) and varies according to ratios and plasma concentrations of lipoproteins, especially VLDL, which is a major acceptor of HDL-CE under hypertriglyceridemic conditions.(25) The main target of PLTP, discovered as a protein that transfers PL among lipoproteins and erythrocytes,(27) is HDL;(28) PLTP also mediates slow fusion of HDL with the concomitant release of apo AI.(20,28–30) Whether the apo AI is lipid-free or just lipid-poor has not been established with certainty; that apo AI binds to lipid surfaces favors the model in which apo AI is released as a lipid-free protein. LCAT transfers the SN-2 acyl chain of phosphatidylcholine to FC and in the absence of FC, LCAT has phospholipase A2 activity; both activities are activated by apo AI.(31) This reaction is important because it converts FC, which has high bioavailabililty, i.e., transfers among lipoprotein and membrane surfaces on a time frame of minutes, to CE, which is transferred by CETP with $t_{1/2}$ ~5 h.(26) Early reports showed that cellular extraction of HDL-CE by SR-B1 occurs without HDL-protein uptake.(32) SR-B1 also extracts FC, PL, and TG albeit at different rates compared to CE (=1)—1.6, 0.2, and 0.7 respectively,(33) and gradually "nibbles" HDL to smaller and smaller remnants on a time frame of hours.(12) Most HDL-modifying activities,(19–21,23,28) including SR-B1,(12) release lipid-free apo AI. The release of lipid-free apo AI is due to its intrinsic instability.(34–37) The similar energetics for apo AI release from HDL and exchange among HDL (38) suggests that apo AI desorption is the rate-limiting step in HDL disruption. Given that lipid-free apo AI is not readily detected in freshly isolated plasma we surmise that lipid-free apo AI formed in vivo either reassociates with HDL or is rapidly relipidated by cell surface ABCA1 for additional nHDL production.

HDL Metabolism

Although most discussions of HDL metabolism are framed within the current model for trans-hepatic RCT (Figure 1), some studies have shed doubt on its validity: HDL-[³H]FC clearance and hepatic uptake in mice is rapid, $t_{1/2}$ = 3.2 min, and is even shorter, $t_{1/2}$ = 1.82 min, in mice over expressing SR-B1;(39). Only ~2% of plasma FC is esterified over 3 min, thus most FC plasma clearance occurs without the esterification step that is implicated in the conventional trans-hepatic RCT model. Considering that human metabolism is slower than that of mice, plasma HDL[³H]FC clearance kinetics in humans is similar (Table 1); most plasma HDL-FC is cleared with $t_{1/2}$ ~8 min, during which <5% of FC is converted to CE;

(40–42) plasma HDL-[^{3}H]FC transfer to bile reaches its maximum value within 40 min,(43) a time frame in which little HDL-FC esterification occurs.

nHDL Metabolism

nHDL obtained by incubating apo AI ABCA1-expressing cells has a higher FC content than HDL (60 vs. 10 mol%, where mol% FC = 100% x moles FC/(moles FC + moles PL)), (1,8,22,44,45) likely reflecting the importance of ABCA1 in reducing cellular cholesterol burden. Although nHDL-[^{3}H]FC, [^{14}C]PL, and [^{125}I]apo AI transfer to different lipoproteins at different rates in vitro, at equilibrium their distribution reflects their natural occurrence within plasma lipoproteins.(1) In mice, the rates of transfer of plasma nHDL- and HDL-FC are similar, $t_{1/2}$ ~5 min and ~2 min respectively; for apo AI $t_{1/2}$ ~460 min.(39) During the ~5 min required for hepatic nHDL-FC uptake, only 2% of plasma FC was converted to CE. Thus, like human and mouse HDL-FC, nHDL-FC is cleared at a rate that is two orders of magnitude faster than that of apo AI and over a time interval in which little FC is esterified —LCAT plays a minor role in nHDL-FC clearance. The rates of HDL- and nHDL-FC clearance are comparable to those for spontaneous desorption and transfer of HDL-FC to LDL, 2–4 min.(15) This mechanism may in part underlie the rapid hepatic FC uptake. However, overexpression of SR-B1 in mice increases the rate of hepatic uptake of plasma HDL-FC.(39) Collectively, these data support a revised RCT model (Figure 2) in which nHDL-FC transfers to HDL after which it is cleared via hepatic SR-B1. However, contributions of direct FC transfer from nHDL spontaneously or via SR-B1 cannot be excluded.

In mice, plasma nHDL-PL transfers to the liver with $t_{1/2}$ ~2 min. This is unexpected given that spontaneous PL transfer halftimes are >1 hour.(15,18) Other mechanisms may be involved. One is SR-B1-mediated uptake although the rate of PL uptake is only 10% of FC uptake in SR-B1-expressing cells.(33) Another is direct transfer to cells at the site of contact between the plasma membrane and nHDL. Although PLTP increases PL transfer from nHDL to human Huh7 hepatocytes, a model cell line of human hepatocytes,(1) the increase (+100%) is still too small to account for the rapid in vivo uptake. Lastly, phospholipolytic activities convert PL to FA and *lyso*PL, which spontaneously transfer between lipoprotein and membrane surfaces with $t_{1/2}$ ~ 3 msec.(16) Current data support a model (Figure 2) in which nHDL-PL transfers to the liver by spontaneous and PLTP-mediated mechanisms that likely involve SR-B1.(1)

Metabolic Segregation of Trans-Hepatic RCT

The plasma clearance kinetics of HDL- and nHDL-FC, PL, and apo AI have similar trends in mice and humans (Table 1): FC and PL disappear within a few minutes whereas apo AI persists for hours and days for mouse and man respectively. During the 5.8 d required for apo AI clearance from human plasma, CE is cleared 6 times and FC is cleared ~10,000 times. Following their initial rapid hepatic uptake, nHDL-FC and PL distribute over all major tissue sites, especially erythrocytes, while most apo AI remains in the plasma compartment.(1) The initial RCT step is cellular FC efflux to apo AI or HDL from whence it transfers to other lipoproteins and ultimately equilibrates with a total body FC pool which is

in a stationary but dynamic state: Stationary because over time there is little change in the plasma concentrations of HDL-apo AI, FC, TG, CE, and PL; dynamic because HDL FC and PL rapidly enter and leave the plasma compartment and exchange with other lipoproteins and tissues, albeit at different rates. Most apo AI associates with HDL but is transferred to the lipid-poor (free?) form by several plasma activities(12,19–21,23,46) making it available for additional cycles of tissue-cholesterol efflux.

SR-B1$^{-/-}$ Mice: An Extreme Model of Dysfunctional HDL and Lipotoxic FC Bioavailability

SR-B1$^{-/-}$ mice have aberrant platelet and erythrocyte morphology and function, and susceptibility to diet-induced atherosclerosis, despite having a high plasma HDL-C concentration;(47,48) moreover, female SR-B1$^{-/-}$ mice are infertile.(49) The cause of these pathophysiologies has been attributed to a dysfunctional HDL for which a molecular mechanism has not been identified. Current evidence suggests that the defect is high FC bioavailability. Compared to WT mice, SR-B1$^{-/-}$ mice have ~2-fold higher plasma HDL levels, (50,51) and more HDL surface as FC (60 vs. 15 mol%);(22) these two attributes are expected to increase HDL-FC bioavailability. A high mol% HDL-FC alone is not atherogenic. However, giving SR-B1$^{-/-}$ mice a high fat, high FC diet increases plasma HDL concentrations and, while maintaining a high mol% HDL-FC, induces atherosclerosis. Thus, even though chow-fed SR-B1$^{-/-}$ mice have several serious metabolic abnormalities, atherogenic HDL in SR-B1$^{-/-}$ mice requires both high mol% FC and high plasma HDL concentration, which would be expected to increase FC bioavailability in a way that increases FC transfer to all cells in contact with plasma. The increased HDL-FC bioavailability overwhelms whole-body cholesterol clearance capacity thereby producing a pathological state in erythrocytes, platelets, the arterial wall, and the ovaries (of female mice). Some dysfunctionality can be reduced by lowering plasma HDL levels and/or mol% FC. In SR-B1$^{-/-}$ mice, probucol, which reduces plasma HDL levels and HDL-FC content, (52) rescues fertility and prevents atherosclerosis.(49,53) Compared to a control diet, dietary β-cyclodextrins, which remove FC from membranes(54) and lower plasma cholesterol (−35%),(55) reduce atherosclerotic plaque size and FC burden in a mouse model of atherosclerosis, the apo E−/− mouse on a high fat, high cholesterol diet.(56)

Other studies in humans are supportive but not axiomatic. A high HDL-cholesterol/PL ratio is atherogenic; patients with the highest ratio of HDL-C/particle had increased carotid atheroprogression compared with those with the lowest ratios, and increase in plaque area over time was greater in those with the highest vs. lowest HDL-C/particle ratio.(57) Without invoking the concept of FC bioavailability, the authors averred that the combination of cholesterol content (mol% FC) and particle number (HDL concentration) better predicted HDL atherogenicity than either parameter taken alone. Similar studies(58,59) are supportive but inconclusive because they did not distinguish between HDL-FC and CE so that FC bioavailability could not be surmised. There are notable exceptions:(60) For example, patients with familial hypoalphalipoproteinemia or familial apo AI/CIII deficiency, both with very low HDL levels, present with the onset of coronary artery disease at ages <40 and <50 years respectively. Tangier patients, who also have low HDL-C, present with coronary

artery disease at age <60 years. Thus, FC bioavailability does not explain everything and other unknown HDL attributes or comorbidities are likely atherogenic.

Transintestinal Cholesterol Excretion (TICE) and FC Bioavailability

Early studies indicated FC transfer to the feces occurs independent of bile;(61,62) FC appears in the feces of dogs with total bile diversion.(63) Studies in mice with deleted ABCG5/ABCG8, the hepatic FC transporter that transfers FC into bile, revealed the underlying mechanism.(64) These mice have biliary cholesterol secretion rates near nil but high fecal neutral sterol levels.(65) Moreover, fecal sterol excretion of $Cyp7A1^{-/-}$ mice is two-times that of WT mice.(66) These findings implicate an alternative route for FC excretion, direct TICE.

The molecular steps associated with TICE are not known. In the context of FC bioavailability there are two likely mechanisms. One is that TICE is mediated by cholesterol transporters located at the plasma membranes at various interfaces between the plasma compartment and the intestine. One likely candidate, SR-B1, which mediates bidirectional FC transport,(67) has been studied.(68) Although intestinal SR-B1 levels correlate with the TICE rates, TICE was actually higher in $SR-B1^{-/-}$ vs. WT mice, perhaps due to the diversion of FC from hepatic disposal to plasma. The other mechanism is spontaneous transfer from plasma lipoproteins to multiple tissue sites including intestine. The FC transfer halftime from HDL to lipoprotein and membrane surfaces is ~5 min.(15) FC translocation across membranes is faster, <1 sec.(69–71) Thus, TICE might begin with FC transfer from lipoproteins to membranes in contact with the plasma compartment followed by diffusion to numerous phospholipid-containing loci between plasma and the intestine. To have net transfer to excretion, there must be an irreversible mechanism that sequesters FC. Given that intestinal PL content is a positive regulator of TICE and that PL are the cholesterophilic component(72,73) of bile, a high concentration of intestinal bile-phospholipid would support net RCT via TICE. Dietary modifications that maintain a high bile-phospholipid content might stimulate TICE, thereby enhancing whole body FC disposal. Ezetimibe, which blocks intestinal FC absorption, doubles the flux of plasma-derived FC into fecal neutral sterols and increases total fecal neutral sterol excretion, plasma CE clearance, and plasma de novo-synthesized cholesterol.(74) Ezetimibe also increases plasma-to-feces FC transfer and elimination via TICE 4-fold.(74) Thus, ezetimibe could also maintain a positive FC gradient between the intestinal lumen and the rest of the body. However, in the context of statin co-therapy, the effect of ezetimibe on the reduction of major vascular events was not different from that of an equivalent reduction in the LDL-C concentration by a higher statin dose.(75) A therapeutic role for ezetimibe via TICE might be verified if its effects on CVD event reduction could be inversely correlated with plasma FC, HDL-FC, and LDL-FC concentrations.

Conclusions

RCT comprises both trans-hepatic and trans-intestinal pathways in which nHDL- and HDL-C are transported as FC. Although HDL-CE is also cleared by trans-hepatic RCT, this is a minor pathway for HDL in humans and mice and for nHDL in mice. Based on current

knowledge, trans-intestinal RCT is likely mediated by spontaneous transfer whereas trans-hepatic RCT is mediated by spontaneous and SR-B1-mediated transfer. In vitro spontaneous PL transfer and in vivo PL clearance rates decrease with increasing acyl chain length suggesting that PL clearance is mediated, in part, by spontaneous transfer. nHDL-apo AI transfers solely to HDL in vivo and several plasma activities elicit the release of lipid-free apo AI from an unstable HDL for additional RCT cycles. Thus, nHDL-FC, PL, and apo AI are metabolically segregated and do not enter a common HDL pathway. While CETP and PLTP modify HDL composition in vitro, in vivo they are minor contributors to trans-hepatic RCT. Deletion of the HDL receptor, SR-B1, is associated with dysfunctional HDL and multiple pathologies for which the underlying cause is excess FC bioavailability. Although current evidence suggests that a high mol% HDL-FC and a high plasma HDL concentration likely contribute to HDL dysfunction, this rule is not axiomatic, suggesting there are other determinants of HDL dysfunctionality. Independent of HDL concentration and mol% HDL-FC, HDL dysfunctionality can be due to myeloperoxidase-mediated apo AI oxidation,[76] a high content of triglyceride,[77] or apo CIII content,[78] and paraoxanase-1-,[79] or apo M-deficiency.[80] One or more of these HDL qualities are found among patients with type 2 diabetes, hypertriglyceridemia,[81] and HIV-positive status after receiving anti-retroviral therapy.[82] Future therapeutic strategies for managing atherogenic HDL should consider the roles of both the trans-hepatic and trans-intestinal RCT pathways. In summary, we propose that the focus of cholesterol clearance, and RCT, should shift to FC, and homeostasis of FC bioavailability, rather than the current focus on HDL-total cholesterol and CE.

Collectively, our studies and those of others have led to a revised RCT model as follows:

- nHDL-FC and human and mouse HDL-FC are cleared faster than apo AI and HDL-CE.

- FC esterification by LCAT plays a minor role in RCT.

- In mice, plasma nHDL-PL is rapidly transferred to the liver via a PLTP-dependent mechanism that involves both spontaneous and SR-B1-mediated paths.

This revised RCT model provokes new mechanistic questions about the role of dysfunctional HDL in impaired RCT.

- Is high plasma FC bioavailability a marker of CVD risk; if so, how may this be lowered therapeutically?

- Mol% HDL-FC and plasma HDL levels may contribute to dysfunctional HDL; do these attributes appear in the plasma HDL of CVD patients?

- Sequestration of FC in the intestine is expected to support the TICE pathway; are there dietary or pharmacological means of TICE enhancement that reduce plasma HDL-FC and does reduction of plasma total cholesterol also reduce mol % HDL-FC?

Addressing these and other questions as they emerge will refine the model of RCT in ways that will better guide the design of new therapies for atheroprotection.

Acknowledgments

Abbreviations

ABCA1	ATP-binding cassette subfamily A, member 1
ACVD	Atherosclerotic cardiovascular disease
Apo	Apolipoprotein
SR-B1	Scavenger Receptor Class B, Type 1
RCT	reverse cholesterol transport
LCAT	lecithin:cholesterol acyltransferase
FC	Free cholesterol
CE	Cholesteryl ester
nHDL	nascent high density lipoproteins
LDL	Low density lipoproteins
FA	Fatty acid
PL	Phospholipid
CYP7A1	Cholesterol-7-α-hydroxylase
CETP	Cholesteryl ester transfer protein
PLTP	Phospholipid transfer protein
TG	Triglyceride
WT	Wild type
Tg	transgenic

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Highlights

- nHDL-FC and human and mouse HDL-FC are cleared faster than apo AI and HDL-CE.

- FC esterification by LCAT plays a minor role in RCT.

- Mouse plasma nHDL-PL rapidly transfers to the liver via a PLTP-dependent mechanism.

- Mol% HDL-FC and plasma HDL levels may contribute to dysfunctional HDL.

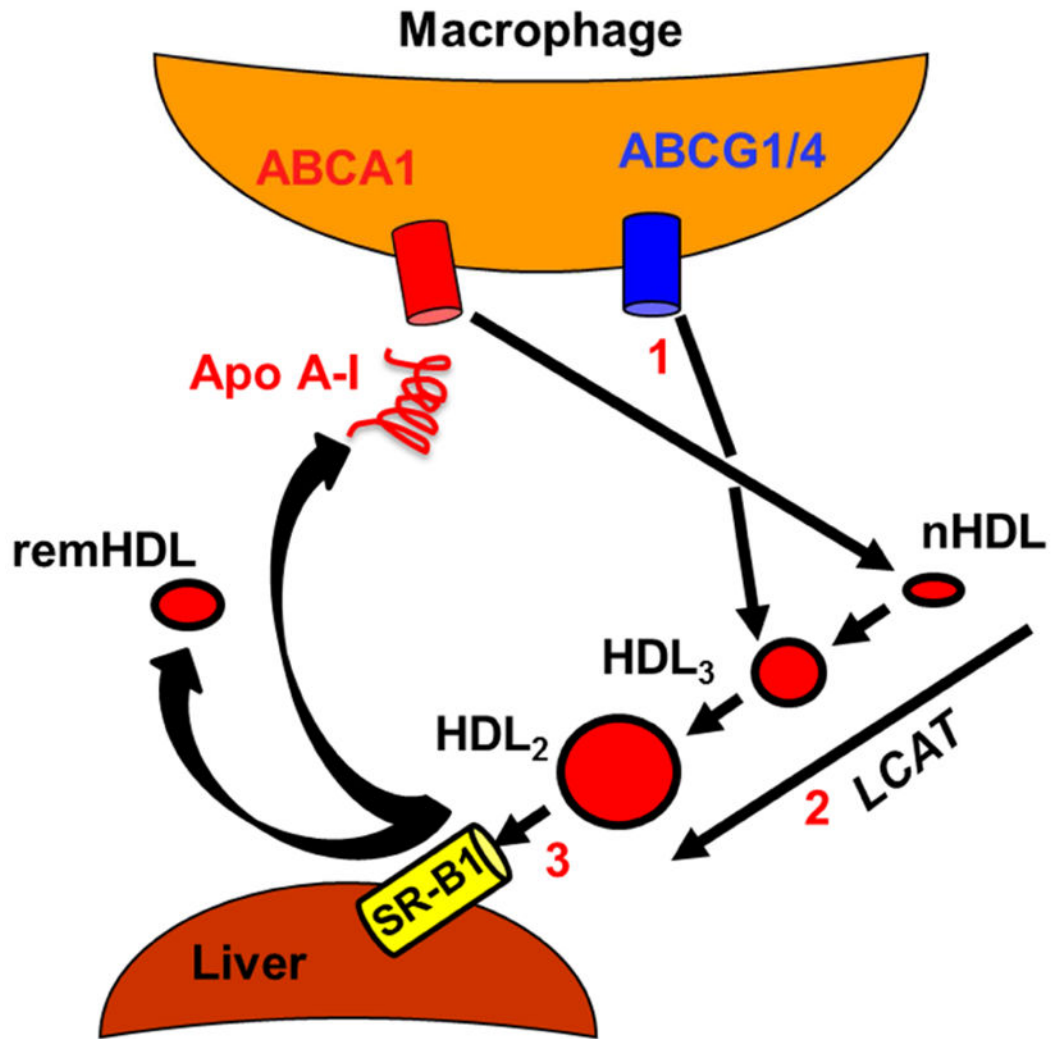- High plasma FC bioavailability may be a marker of CVD risk.

Figure 1.
Traditional Model of RCT in the Context of Atheroprotection (Adapted from Glomset and Ross).(2) 1) FC efflux from macrophages initiates the formation of discoidal nHDL, which contains FC, PL, and apo AI. 2) LCAT catalyzes the conversion of FC to CE, which forms a central core within spherical HDL. 3) SR-B1 selectively extracts lipids, especially FC and CE, from the mature HDL particle leaving an apo-rich remnant HDL (remHDL) particle and lipid-free apo AI that returns to another RCT cycle.
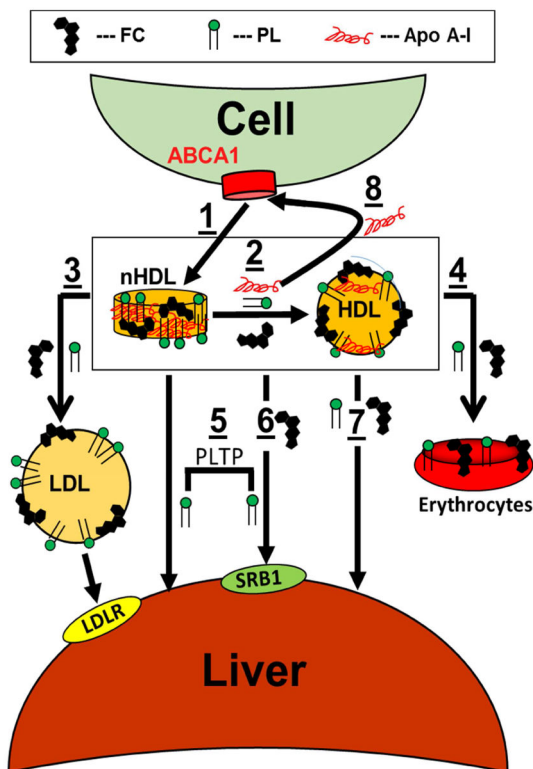
Figure 2.
Revised RCT Model. ABCA1-expressing cells extrude FC and PL via the interaction of apo AI with ABCA1 giving nHDL (1). nHDL-apo AI and some nHDL-FC and PL rapidly transfer to HDL, $t_{1/2} < 2$ min (2); concurrently some nHDL-FC and PL transfer to LDL (3). Over the same time frame, nHDL- and HDL-FC and PL transfer mainly to the liver (5, 6, 7) while some nHDL-apo AI is recycled to ABCA1 (8). Over time, FC and PL equilibrate among erythrocytes (4), extra-hepatic tissues, and lipoproteins. nHDL- and HDL-FC and PL accretion in the liver occurs via spontaneous transfer and SR-B1 (5, 6, 7), the latter being promoted by PLTP (6). Modified from Xu et al. (1).

Table 1

Kinetics of Clearance of HDL and nHDL [*]

	Human Plasma Lifetime		Mouse Plasma Lifetime	
	$t_{1/2}$	FCR	$t_{1/2}$	FCR
HDL-apo AI	5.8 d (83)	0.12/d	5.8 h (84)	0.12/h
HDL-FC	7–8 min (40)	0.09/min	3.2 min (39)	0.22/min
HDL-CE	0.92 d (85)	0.75/d	3.3–3.5 h(40,42,43,85)	0.21/h
nHDL-FC	-		5.2 min (1)	0.13/min
nHDL-PL	-		2.0 min (1)	0.35/min
nHDL-apo AI	-		7.7 h (1)	0.09/h

[*] Fractional catabolic rate (FCR) = $\ln 2/t_{1/2}$.