Reverse cholesterol transport dysfunction is a feature of familial hypercholesterolemia

Joan Carles Escolà-Gil[1,2,3†] orcid.org/0000-0001-9021-2485, Noemí Rotllan [1,2] (https://orcid.org/0000-0002-0587-8045), Josep Julve[1,2,3†] (http://orcid.org/0000-0002-6531-2246), Francisco Blanco-Vaca[2,3,4] (https://orcid.org/0000-0001-7380-5385)

[1] Institut de Recerca de l'Hospital de la Santa Creu i Sant Pau, Institut d'Investigacions Biomèdiques (IIB) Sant Pau, Barcelona, Spain

[2] CIBER de Diabetes y Enfermedades Metabólicas Asociadas, CIBERDEM, Madrid, Spain

[3] Departament de Bioquímica i Biologia Molecular, i Universitat Autònoma de Barcelona, Barcelona, Spain

[4] Servei de Bioquímica, Hospital de la Santa Creu i Sant Pau, Institut d'Investigacions Biomèdiques (IIB) Sant Pau, Barcelona, Spain

Address correspondence to: Joan Carles Escolà Gil (jescola@antpau.cat) or Josep Julve (jjulve@santpau.cat)

Other author e-mails:

fblancova@santpau.cat, nrotllan@santpau.cat

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Structured abstract

Purpose of review: We seek to establish whether high-density lipoprotein HDL metabolism and reverse cholesterol transport (RCT) impairment is an intrinsic feature of familial hypercholesterolemia (FH).

Recent findings: RCT from macrophages (m-RCT), a vascular cell type of major influence on atherosclerosis, is impaired in FH due to defective low-density lipoprotein receptor (LDLR) function via both the HDL- and LDL-mediated pathways. Potential mechanisms include impaired HDL metabolism, which is linked to increased LDL levels, as well as the increased transport of cellular unesterified cholesterol to LDL, which presents a defective catabolism.

Summary: RCT dysfunction is a consistent finding in the literature and thus an intrinsic feature of mutation-positive FH linked to decreased HDL levels as well as impaired HDL remodeling and LDLR function. It remains to be explored whether these alterations are also present in less well-characterized forms of FH, such as cases with no identified mutations, and whether they are fully corrected by current standard treatments.

ABBREVIATIONS

ABC, ATP binding cassette transporter

APO, apolipoprotein

CE, cholesterol ester

CEC, cholesterol efflux capacity

CETP, cholesteryl ester transfer protein

CVD, cardiovascular disease

FH, familial hypercholesterolemia

HDL, high-density lipoprotein

HDL-c, HDL cholesterol

LCAT, lecithin:cholesterol acyltransferase

LDL, low-density lipoprotein

LDL-c, LDL cholesterol

LDLR, LDL receptor

LXR, liver X receptor

miRNA, micro RNA

m-RCT, macrophage-specific reverse cholesterol transport

PCSK9 proprotein convertase subtilisin/kexin type 9 PLTP, phospholipid transfer protein

RCT, reverse cholesterol transport

SR-BI, scavenger receptor BI

SREBP, sterol response element-binding protein

TICE, transintestinal cholesterol excretion

UC, unesterified cholesterol

63 VLDL, very low-density lipoprotein

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Introduction

Familial hypercholesterolemia (FH) is classically defined as an autosomal codominant disease characterized by elevated plasma low-density lipoprotein (LDL) cholesterol (LDL-c) and a high risk of premature cardiovascular disease (CVD) [1]. It is mainly due to the loss of function variants in the LDL receptor gene (*LDLR*). FH cases with no detected *LDLR* mutations might be due to pathogenic variants in other genes encoding proteins that interact with the LDLR, such as the LDLR ligand, apolipoprotein B-100 (*APOB*), and proprotein convertase subtilisin/kexin type 9 (PCSK9). Recently, some studies using different weighted LDL-c gene scores showed that 20 to 80% of mutation-negative FH patients exhibited a high score, suggesting potential forms of polygenic FH [2, 3].

Recent reviews have critically addressed the role of high-density lipoprotein (HDL) in atherosclerosis development, highlighting the potential rapid movement of unesterified cholesterol (UC) from cells and triglyceride-rich particles to HDL as well as from HDL to LDL or tissues [4, 5]. There is also evidence indicating that HDL remodeling, metabolism, and function, including its ability to induce macrophage cholesterol efflux, are impaired in the monogenic forms of FH [6-10]. Whether this impairment is intrinsic to the disease and influences the entire reverse cholesterol transport (RCT) pathway and cardiovascular risk and is corrected by current treatments is not yet well-established.

HDL content and remodeling alterations in FH

The HDL particle composition is determined by the presence of a diversity of proteins, enzymes, lipids, and microRNAs (miRNAs) that confer specific functions to HDL. The physicochemical composition of HDL may vary and determine the particle shape, density, size, charge, and biological activity. Indeed, HDL composition is a key atheroprotective determinant. Decreased levels of HDL-cholesterol (HDL-C) have been consistently observed in both heterozygous FH and homozygous FH in association with HDL structural and functional abnormalities [6-10].

In an early study of heterozygous FH patients, HDL particles were found to be smaller than those of control subjects [11]. Patients with type IIa hypercholesterolemia displayed reduced HDL3 production and enhanced fractional catabolic rates concomitantly with decreased apolipoprotein (APO) A1 (APOA1) levels [9, 12, 13]. Additionally, FH patients exhibited elevated concentrations of small nascent preβ1-HDL particles [9, 14, 15] but reduced levels of large HDL2 particles when compared to normolipidemic subjects [9]. Other studies—but not all [8]—have demonstrated hypoalphalipoproteinemia due to increased catabolic rates in FH [16, 17]. Interestingly, elevations in plasma APOE have been reported in FH patients [13, 18], and the *APOE* genotype might influence the plasma HDL-C levels in these patients [19]. Another apolipoprotein differentially expressed in FH is APOL1, which has been proposed as a predictor of CVD events and mortality [20]. Additionally, a decrease in lecithin:cholesterol acyltransferase (LCAT) content in FH patients suggested significant modifications of HDL atheroprotective properties [20]. Indeed, the HDL3 particles of FH patients have been reported to have diminished antioxidant and anti-inflammatory

functions [12]. HDL particles from FH patients are enriched with cholesteryl esters (CE), depleted in phospholipids, and have an increased sphingomyelin/phosphatidylcholine ratio [9]. In a recent study from our group, different lipid transfer proteins and enzymes associated with HDL remodeling were evaluated in non-treated FH patients with an identified *LDLR* mutation and compared to normolipidemic patients similar in age [14]. The adult FH patients had lower levels of APOA1 and HDL-C but higher HDL APOA2 and APOE content. Interestingly, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities were found to be higher in the non-treated FH patients along with reduced LCAT activity [14]. Therefore, these changes might explain, at least in part, the increased content of preβ-HDL particles as well as the decrease in the amount of mature HDL particles in FH [14]. All these changes were also found in LDLR-deficient mice as well as in human APOB100 transgenic mice (it is noteworthy that mice do not express CETP) [21]. The latter mouse model also exhibited hypercholesterolemia due to elevated LDL but also a functional LDLR, indicating that hypercholesterolemia might be directly linked to altered HDL remodeling independently of CETP and LDLR. Although the mechanisms involved in these biochemical changes are largely unknown, they could be related to the accumulation of cholesterol-derivatives activating the liver X receptor (LXR), which may increase CETP and PTLP gene expression [22, 23]. We suggest that increased CETP may not promote RCT in cases with reduced LDLR function, and increased PTPL-mediated remodeling may not be effective in generating more mature HDL particles in the context of low LCAT action.

HDL- and plasma-mediated macrophage cholesterol efflux is impaired in FH

The cholesterol efflux capacity (CEC) of HDL represents the first step in the reverse cholesterol transport (RCT) pathway. This functional property of HDL has been associated with atherosclerotic cardiovascular disease independently of HDL-C [24, 25]. As previously mentioned, compelling evidence supports the notion that an altered HDL metabolism and remodeling underlies a defective CEC mediated by HDL in FH patients. Indeed, HDL2 isolated from the plasma of FH patients presented a lesser CEC from cholesterol-loaded macrophages, which was mediated through both scavenger receptor class B type I (SR-BI) and ATP-binding cassette (ABC) transporter G1 (ABCG1), when compared to non-FH subjects [9]. In this same study, HDL3-mediated, SR-BI-dependent (but not ABCG1) CEC was also reduced [9]. In line with these findings, FH-derived HDL3 with a high triglyceride content also showed a reduced CEC from lipid-loaded macrophages in an independent study [26]. The reduced concentrations of HDL2 and HDL3 [9, 12], along with the altered activities of remodeling enzymes and lipid transfer proteins [14], may conceivably explain the lower CEC rates promoted by APOB-depleted plasmas or serums in both treated and untreated FH patients [14, 27]. It is noteworthy that CEC of APOB-depleted serum was inversely and independently associated with the carotid intima-media thickness of FH patients treated with a lipid-lowering therapy, although this association was weaker following adjustment for HDL-C and APOA1 levels [27]. We also reported a significant reduction in the amount of macrophage-derived cholesterol accumulated in HDL when the CEC of FH plasmas was evaluated [21]. Taken together, these observations identify the significant

alteration of HDL-mediated CEC as a feature of FH patients with signs of atherosclerotic cardiovascular disease—even after adjustment for classical risk factors.

Several large prospective studies have measured CEC promoted by either APOB-depleted serum or plasma as a surrogate of HDL functionality related to the risk of atherosclerotic cardiovascular disease [24, 25, 28]. However, the CEC of APOB-depleted serum does not always correlate with that of the whole serum, which is likely because the latter contains more physiological cholesterol acceptors [29]. Significant evidence indicates that LDL also contributes to macrophage CEC [30]. We recently reported that CEC induced from the plasma of untreated FH patients (with an identified *LDLR* mutation) was significantly impaired compared to that of normolipidemic volunteers. Further, our results also indicated that isolated LDL particles were major acceptors of the macrophage-derived radiolabeled cholesterol in both normolipidemic and FH plasmas [21]. Importantly, most of the transferred cholesterol in the LDL was unesterified, thereby indicating the marginal role of CETP and LCAT in this process [21]. Overall, these observations reveal that LDL enhances the efflux of cholesterol directly and indirectly by acting as a sink for cholesterol released from cells by HDL, which is in line with previous observations [30]. However, the transport of cholesterol from HDL to LDL and its slow return into the circulation for LCAT esterification (if the LDL is not previously removed from the circulation by the cell LDLR, as is often the case in FH) may not be representative of the exchanges that happen at the arterial intima level. Indeed, at this location, LDL is trapped by interactions with proteoglycans, and the UC transferred into LDL would likely accumulate at the intima lesion sites [30].

CETP could also be driving CE transfer from HDL to LDL in the plasma of FH patients, promoting the formation and vascular accumulation of atherogenic LDL [31]. Very low-density lipoproteins (VLDL) lipolysis, albumin, and a number of enzymes and transfer proteins activities may also be relevant factors associated with plasma CEC [32, 33]. While VLDL do not show significant CEC [21], the transfer of UC from triglyceride-rich lipoproteins to HDL during lipolysis could compete with that from macrophages [4].

Taken together, plasma elevations of LDL-C in FH subjects would appear directly related to the existence of dysfunctional HDL, which are characterized by both an altered remodeling and impaired CEC from macrophages. However, such a decreased cellular cholesterol efflux could also be in part due to a lower macrophage transporter activity. Indeed, *ABCA1* expression has been shown to be downregulated in the monocytes of FH patients with genetic defects in *LDLR* [34]. In this regard, compelling evidence suggests that these transporters also can be regulated by microRNAs (miRNA). Also, many studies over the past years have identified miRNAs as important regulators of HDL metabolism [35, 36]. Furthermore, it has been shown that miRNAs can be transported in the plasma and delivered to recipient cells by HDL, thus directly influencing gene expression [37]. The most abundant miRNAs associated with HDL in FH patients are miR-223, -105, and -106a [37]. The incubation of human cultured hepatocytes (Huh7 cells) with HDL isolated from FH patients, that contained increased miR-223 and miR-105 levels, induced downregulation of several of their putative target genes [37]. Furthermore, in silico target prediction identified mRNAs that were conserved putative target of 22 differentially abundant miRNAS on FH HDL [38, 39]. In addition to its anti-inflammatory role, miR223 may influence systemic and hepatic

cholesterol metabolism. Indeed, miR233 overexpression attenuates SR-BI protein expression and liver HDL-C uptake [40]. Consistently, the genetic ablation of miR-223 enhances hepatic SR-BI expression and HDL-C uptake [41]. Interestingly, miR-223 also prevents cholesterol biosynthesis through directly repressing the sterol enzyme, 3-hydroxy-3-methylglutaryl-coA synthase 1 (*HGMCS1*), expression [41]. Moreover, the overexpression of miR-223 also enhances ABCA1 expression in Huh7 cells, and thus promotes cholesterol efflux through the repression of transcription factor Sp3. Overall, these studies highlight the role of miR-223 in regulating cholesterol homeostasis, albeit further studies are needed to specifically establish its role in regulating FH macrophage transporters.

On the other hand, several studies measuring circulating miRNAs in FH children have shown that miR-33a/b and miR-200c were upregulated [42, 43]. However, whether these circulating miRNAs were associated with HDL has not been reported [44-46]. Like many intronic miRNAs*, miR-33a* is co-transcribed with its host gene sterol response element-binding protein (*SREBP)* 2, which targets genes involved in cholesterol export, including *ABCA1*.

m-RCT is impaired in FH caused by LDLR-mediated function

The entire RCT transport from cells, such as macrophages, loaded with radiolabeled cholesterol to feces, also called macrophage-specific RCT (m-RCT), has been assessed in experimental animals. Genetically modified mice resembling a human monogenic FH mutation, usually in homozygosis, have been used in these experiments. We recently found that [^{3}H]cholesterol derived from labeled macrophages injected into the peritoneal cavity of FH mouse models (i.e., LDLR-deficient mice or PCSK9-overexpressing mice) was rapidly transferred to the hypercholesterolemic plasma and mostly associated with LDL. However, the fecal excretion of macrophage-derived cholesterol was significantly impaired in these mice, thereby indicating that that LDLR was essential in supporting the last step of the m-RCT route [21]. In line with these findings, a reduction in the liver and adrenal gland uptake of radiolabeled CE in HDL was previously noted in LDLR-deficient mice [47]. More importantly, both radiolabeled LDL- and HDL-cholesterol showed an impaired clearance in LDLR-deficient mice, and this was concomitant with a lower transfer of cholesterol from both lipoproteins to the feces [21]. Although these mice were homozygous lacking LDLR, there is no reason to believe that the defective m-RCT would not occur in heterozygous animals—albeit presumably at a lower scale. In contrast, the m-RCT rate remained unchanged in human APOB100 transgenic mice with fully functional LDLR despite increased levels of plasma APOB-containing lipoproteins and a higher accumulation of macrophage-derived cholesterol in the LDL fraction [21]. Since the APOB100 transgenic mice presented with HDL remodeling impairment, it can be suggested that LDLR is needed to maintain m-RCT rate.

Overall, these findings strongly indicate that monogenic FH due to LDLR functionality impairment in mice does present with defective m-RCT. FH patients may present a more intense impairment, because their CETP activity could enhance the transfer of HDL-CE (formed by the LCAT action) to LDL and be subjected to a slower transfer to the liver. Indeed, CETP enhanced the amount of macrophage-derived cholesterol in LDL and the overall flux of [^{3}H]cholesterol to the feces in mice, but failed at promoting the m-RCT rate in the absence of LDLR [48].

It should be noted that LDL provides a significant amount of cholesterol for transintestinal cholesterol excretion (TICE) in human and mouse jejunal explants at its basolateral side [49]. Interestingly, TICE was increased in PCSK9-deficient mice whereas decreased upon an acute injection of PCSK9 [49]. However, TICE tended to be higher in LDLR-deficient mice [49], suggesting the activation of alternative compensatory mechanism(s) in conditions of chronic LDLR deficiency.

Experimental and clinical therapeutic strategies

Early reports demonstrated that LDL-apheresis was highly efficient in reducing not only LDL-C in severe FH patients but also large APOE-containing HDL and preβ-HDL particles [50]**.** This transitorily reduced the ability of these plasmas to induce macrophage cholesterol efflux [51]. In line with these findings, a recent report found that HDL from FH patients had increased malondialdehyde-APOA1 adducts, which was in close association with a defective CEC induced by APOB-depleted serum; this functional HDL alteration was not improved by LDL-apheresis [52]. Interestingly, dicarbonyl scavenging with 2-hydroxybenzylamine was able to prevent atherosclerosis

and foam cell formation by improving HDL CEC in LDLR-deficient mice [52]. Several therapeutic approaches have also failed to induce macrophage cholesterol efflux in FH patients. Indeed, lomitapide treatment was found to reduce the amount of large-buoyant HDL and preβ-HDL particles in homozygous FH patients, and, consistently, the ABCA1-mediated CEC of APOB-depleted serum was impaired after this treatment [53]. Furthermore, a recent study reported that an antibody to PCSK9, evolocumab, did not affect either plasma HDL subclasses nor macrophage cholesterol efflux [54]. However, this study was conducted in only three FH patients who had been under apheresis treatment for 11 years [54]. It should be noted that human PCSK9 reduced ABCA1-dependent macrophage cholesterol efflux to APOA1 induced by the activation of the LXR/RXR pathway; this effect was fully abrogated by an anti-PCSK9 antibody or LDLR deficiency [55]. Non-treated FH children displayed alterations in HDL, such as an increase in HDL3-C and large HDL with respect to healthy controls. Interestingly, the smaller HDL particles were enriched in CE and had lower UC and phospholipid content [56]. The amount of very large HDL was normalized in statin-treated FH children [56], but whether this affected macrophage cholesterol efflux is unknown. In this context, we found that statin treatment was not able to normalize the ability of adolescent FH plasmas to induce macrophage cholesterol efflux (unpublished data). However, the lipoprotein distribution of macrophage-derived radiolabeled cholesterol was in part increased in HDL following statin treatment, whereas that of LDL was reduced concomitantly (unpublished data). Of note, rosuvastatin—but not atorvastatin—induced ABCA1-dependent macrophage cholesterol efflux and promoted m-RCT in wild-type mice [57]. Clearly, more studies are needed to clarify the potential of PCSK9 inhibitors

and statins in regulating HDL-mediated macrophage cholesterol efflux and their impact on the entire RCT pathway in FH.

Another therapeutic approach is the injection of recombinant HDL particles, such as CER-001, which has been shown to enhance macrophage cholesterol efflux, fecal cholesterol excretion, and atherosclerosis regression in LDLR-deficient mice [58]. CER-001 reduced the mean vessel wall area measured by magnetic resonance imaging in homozygous FH patients [59], thereby indicating that targeting HDL-mediated cholesterol efflux may represent a successful strategy for regressing atherosclerotic plaque. However, CER-001 did not favorably influence the carotid atherosclerosis of patients with HDL deficiencies despite significant elevations in plasma CEC after CER-001 infusion [60]; whether the activity of this compound in patients is disease-dependent remains to be seen.

Conclusions

There is a notable consensus that points to an altered HDL remodeling and composition and impaired m-RCT in FH. This impairment can be captured, at least in part, in cholesterol efflux experiments in FH patients and the m-RCT experiments in genetically modified mice. These functional alterations have been reported at different steps of the RCT pathway (summarized in **Figure 1**) and seem to be especially dependent on the existence of increased LDL and LDLR function. Whether this FH feature is critical for atherosclerosis development and is ameliorated by current standard treatments for the disease needs to be further investigated. Although the latter could be anticipated considering the potential of current treatments to achieve important reductions in LDL-

C, the data currently available would support a rather incomplete improvement in HDL and CEC in FH patients treated with statins. Future research should also compare the effect of different pharmacological treatments in CEC in both mutation-detected FH and mutation-negative FH cases.

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Human and Animal Rights and Informed Consent

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Figure legend

Figure 1. Macrophage reverse cholesterol transport (RCT) pathway is impaired in familiar hypercholesterolemia (FH). The functional alterations of the HDL-mediated RCT pathway detected at the different steps are based on results from human studies and FH mouse models. The first step of reverse unesterified cholesterol (UC) transport is initiated in macrophage foam cells. FH patients display increased cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities but reduced lecithin-cholesterol acyltransferase (LCAT) activity. These changes are associated with lower α-HDL cholesterol and APOA1 levels along with significant alterations in the α-HDL composition and higher levels of nascent preβ-HDL particles. UC efflux from macrophages to HDL particles, promoted by both the transmembrane cholesterol ATP binding cassette transporters (ABC), A1 and G1, is impaired in FH patients. A significant part of macrophage-derived UC present on the surface of preβ-HDL and α-HDL particles and can be rapidly transferred to LDL into the circulation. This process appears to be independent of CETP. It should be noted that, based on whether cholesterol efflux from macrophages takes place in the arterial intima, LDL could be trapped by interactions with proteoglycans, and the UC transferred into LDL at this location would be finally accumulated at the intima lesion sites. Circulating CETP in FH patients can also drive the transfer of esterified cholesterol (EC) from HDL toward the core of LDL. In experimental models of FH, LDLs, carrying their load of macrophage-derived cholesterol, cannot be correctly internalized by the hepatic LDL receptor (LDLR). Hepatic cholesterol is ultimately secreted into the bile and to the intestine by ABCG5/G8 as UC, thereby completing the hepatobiliary RCT route. Overall, under the genetic

absence of the LDLR, or under conditions leading to its dysfunctionality, the rate of this macrophage-derived UC reverse transport to feces is decreased. Although LDLR-deficiency does not show clear effects on transintestinal cholesterol export (TICE), the acute injection of PCSK9 may regulate TICE *in vivo*.

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