



HDL function and novel HDL-targeted therapies for preventing atherosclerotic cardiovascular disease: From mouse models to human disease

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Resum. Malgrat que la relació inversa entre les concentracions baixes de colesterol associat a lipoproteïnes d'alta densitat i l'increment del risc de patir una malaltia cardiovascular està comunament acceptada, l'ús d'alguns fàrmacs que incrementen les concentracions de colesterol d'HDL no s'ha trobat associat a una reducció en l'aparició d'episodis cardiovasculars. Es considera que el transport revers de colesterol (RCT) és el principal responsable de l'efecte cardioprotector de l'HDL, estimulant el flux de colesterol des dels macròfags de les cèl·lules escumoses en la lesió arterioscleròtica cap a fetge on és eliminat parcialment en femtes. A més del RCT, les HDL tenen altres propietats cardioprotectores degudes als seus efectes antioxidant i antiinflamatori. Els estudis en ratolins modificats genèticament han permès demostrar que aquestes funcions de les HDL estan associades a la susceptibilitat a l'arterioesclerosi i suggereixen que fàrmacs que les puguin estimular podrien ser considerats com a noves estratègies terapèutiques per a prevenir el desenvolupament de la placa arterioescleròtica i el risc d'episodis cardiovasculars. El potencial antitumorogènic de les HDL és una àrea d'investigació recent. Aquests tipus d'investigacions són essencials per a poder traslladar els resultats obtinguts a humans.

Els estudis més recents indiquen que l'augment del flux de colesterol des dels macròfags fins a les HDL, el primer pas del RCT, està inversament relacionat amb l'aparició d'episodis cardiovasculars. Per tant, la majoria d'estudis actuals van enfocats a millorar les propietats cardioprotectores de les HDL, més que no pas a intentar augmentar les concentracions de colesterol d'HDL.

Abstract. Although significant evidence supports the concept that low high density lipoprotein cholesterol (HDL-C) is associated with an increased risk of cardiovascular disease (CVD), the failure of several HDL-targeted therapies to reduce CVD has cast doubts on the HDL-C hypothesis. Reverse cholesterol transport (RCT) is currently thought to be a major HDL cardioprotective property by which HDL promotes cholesterol efflux from macrophage foam cells and delivers that cholesterol to the liver, from where it will be partly eliminated through bile and feces. Beyond RCT, HDL exhibits other cardioprotective properties, such as antioxidant and anti-inflammatory effects. Data from genetically-engineered mice indicate that these HDL functions are closely associated with atherosclerosis susceptibility, thereby suggesting that the promotion of HDL

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functional properties may be considered a novel therapeutic strategy to reduce the atherosclerotic plaque burden and subsequent cardiovascular events. Furthermore, the potential anti-tumorigenic role of HDL is currently under investigation. This research has been essential for trying to translate experimental results obtained in mice to humans. The results of several recent human studies indicate that enhanced macrophage cholesterol efflux, the first step of RCT, is inversely associated with CVD events. Therefore, the current research is focused on improving HDL function rather than simply targeting HDL-C levels.

Abbreviations. HDL: high density lipoprotein; LDL: low density lipoprotein; CVD: cardiovascular disease; CETP: *cholesterol ester transfer protein*; ACAT: *cholesterol acyl transferase*; PLTP: *phospholipid transfer protein*; HL: *hepatic lipase*; EL: *endothelial lipase*; ApoA-I: *apolipoprotein A-I*; ABC: *ATP-binding cassette*; RCT: *reverse cholesterol transport*; LXR: *liver X receptor*; PPAR: *peroxisome proliferator-activated receptor*; NPC1L1: *niemann-Pick C1-Like 1*; CYP7A1: *cytochrome P450 family 7 subfamily 1*; PON1: *paraoxonase/arylesterase 1*; PAF-AH: *platelet-activating factor-acetylhydrolase*; SIRT: *NAD⁺-dependent deacetylase sirtuin*; SERM: *selective estrogen receptor modulators*; SFA: *saturated fatty acids*; PUFA: *polyunsaturated fatty acids*; TAM: *tamoxifen*; RAL: *raloxifene*; TOR: *toremifene*; ER: *estrogen receptor*; HPA: *hypothalamic-pituitary-adrenal*; CRF: *corticotrophin releasing factor*; CORT: *corticosterone*

Introduction

The current concept that high-density lipoprotein (HDL) protects against cardiovascular disease (CVD) is based on abundant preclinical and epidemiological data. Originally, the main explanation was associated with the ability of HDL to induce cholesterol efflux from macrophage foam cells in atherosclerotic lesions (Glomset, 1968). Historically, the macrophage cholesterol efflux has been considered the main HDL antiatherogenic function. However, reverse cholesterol transport (RCT) is a complex pathway and macrophage cholesterol efflux is only considered the first step of the pathway. This antiatherogenic pathway may be regulated at different steps, such as (i) the macrophage cholesterol efflux, (ii) the transport of cholesterol through the plasma compartment, (iii) the liver uptake, (iv) the hepatobiliary excretion, and (v) the excretion from the body (see Figure 1 for details).

The failure of various HDL-targeted therapies to reduce CVD has cast doubts on the HDL hypothesis. Two large trials (ILLUMINATE and dal-OUTCOMES) with cholesterol ester transfer protein (CETP) inhibitors were stopped for disappointing results or reason of futility (Barter and Rye, 2012), and a large Mendelian randomization study showed that a single-nucleotide polymorphism (SNP) in the endothelial lipase (EL) gene did not affect the risk of myocardial infarction despite increasing HDL cholesterol (HDL-C) (Voight et al., 2012). Therefore, the inverse relation between cardiovascular risk and HDL is not strictly related to the mass of cholesterol transported by HDL (Rader and Hovingh, 2014). HDL comprises a complex mixture of heterogeneous lipoproteins ranging from nascent pre β -HDL particles to mature HDL spherical particles. Apolipoprotein (apo) A-I is the main HDL protein and significant evidence reveals that most HDL antiatherogenic properties depend on apoA-I content (Mineo and Shaul, 2012). Among these anti-atherosclerotic properties, the best recognized is the ability of HDL to stimulate efflux of excess cholesterol from macrophage foam cells, and consequently this is the most evaluated mechanism (Camont et al., 2011). This efflux to HDL occurs via different pathways, the most efficient ones being mediated by two cholesterol transporters, transmembrane ATP-binding cassette (ABC): ABCA1 and ABCG1. ABCA1 promotes cholesterol transport to nascent pre β -HDL and lipid free apoA-I, whereas ABCG1 facilitates the efflux to mature α -migrating HDL. Macrophage ABCA1 and ABCG1 form the primary route of cholesterol efflux and, therefore, start the macrophage-to-feces RCT (Figure 1). The results of several recent studies indicate that stimulation of macrophage cholesterol efflux to HDL mediated by ABCA1 transporter is inversely associated with the incidence of atherosclerotic CVD events, thereby indicating the importance of this function as biomarker for predicting CVD risk (Rohatgi et al., 2014; Saleheen et al., 2015).

The efflux of cellular cholesterol to HDL initiates RCT in all tissues, but the fraction that originates from the macrophage foam cells located in the intima is considered the main RCT component directly involved in atherosclerosis (Cuchel and Rader, 2006). An assay for measuring in vivo macrophage-to-feces RCT by tracing the reverse [3H]-cholesterol transport from lipid-laden macrophages to feces in mice was developed (Rader et al., 2009). The method has been used to study the role of different therapies and pathways relevant for RCT and HDL-mediated atheroprotection (Escola-Gil et al., 2009; Annema and Tietge, 2012). However, this method is only applicable in animal models and, for ethical issues, seems impossible to apply in hu-

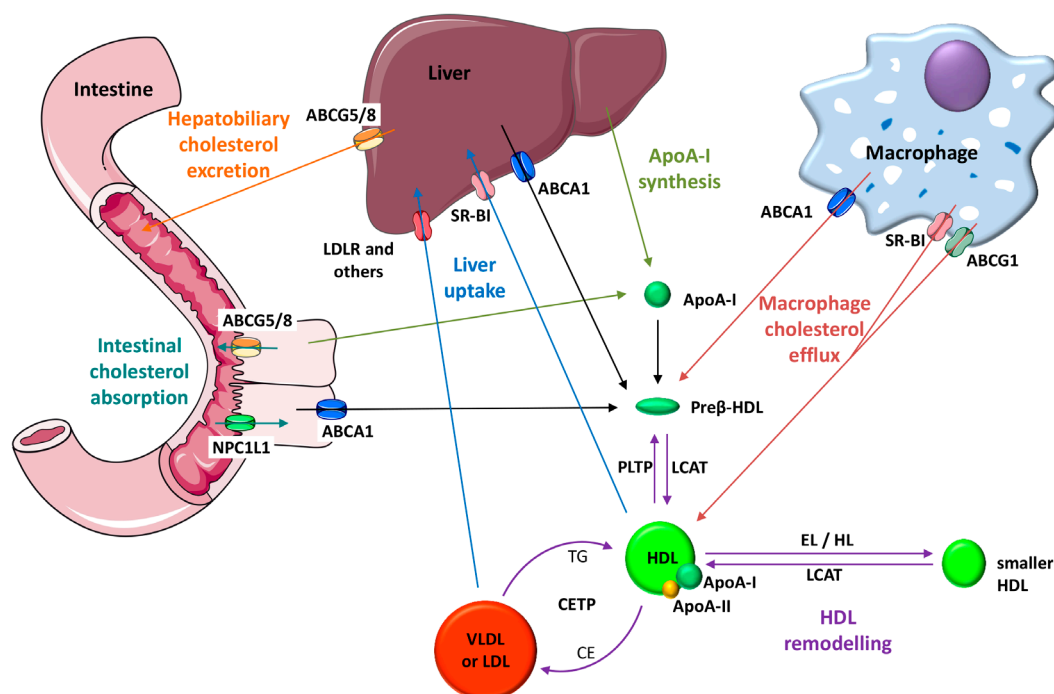


Figure 1 legend. Schematic diagram of reverse cholesterol transport pathway. ApoA-I is synthesized by liver and small intestine, acquires phospholipids and become nascent partially lipidated pre β -HDL particles. Pre β -HDL particles acquire free cholesterol from macrophages via adenosine triphosphate-binding cassette (ABC) A1 transporter. Free cholesterol is converted into cholesteryl ester within the HDL particle by the action of lecithin:cholesterol acyltransferase (LCAT), thereby resulting in mature HDL. ApoA-I is the major HDL protein and activates LCAT, whereas apoA-II, the second HDL protein, displaces apoA-I from HDL particles. The scavenger receptor type BI (SR-BI) and ABCG1 facilitate the cholesterol efflux process from macrophages to mature HDL. Phospholipid transfer protein (PLTP) promotes the transfer of phospholipids and free cholesterol from triglyceride-rich lipoproteins into HDL, producing a remodelling process by which pre β -HDL particles can be generated. Furthermore, PLTP may promote the fusion of smaller HDL particles and subsequent generation of larger HDL particles. Endothelial lipase (EL) and hepatic lipase (HL) hydrolyse HDL triglycerides and phospholipids, thereby converting large HDL particles to smaller ones. HDL-cholesteryl ester can be transferred to VLDL or LDL by cholesteryl ester transfer protein (CETP) and returned to the liver through low-density lipoprotein receptor (LDLR) or other receptors. The liver also selectively takes up HDL-associated cholesterol via SR-BI. The liver ABCG5/G8 heterodimer is involved in cholesterol transport to bile. Niemann-Pick C1-like 1 (NPC1L1) is of crucial importance for absorbing macrophage-derived cholesterol in the small intestine. Cholesterol may also be excreted back to the lumen by the intestinal ABCG5/G8 heterodimer.

mans. Some studies are in process to discover the way to evaluate the entire RCT pathway in humans such as the use of [3H]-cholesterol/albumin complexes or [2,3-13C2]-cholesterol but the application of these assays are still awaited (Schwartz et al., 2004; Turner et al., 2012).

HDL targets

With the creation and detailed analysis of genetically-modified mice, a solid body of new information emerged on the mechanisms controlling the RCT pathway. Indeed, the atheroprotective role of apoA-I has been corroborated in transgenic mice (Rubin et al., 1991). Such beneficial effects of apoA-I on the development of athe-

rosclerosis can be related to its stimulatory effect on macrophage-to-feces RCT, which has been demonstrated both in transgenic mice expressing human apoA-I (hApoA-I) (Zhang et al., 2003) and in wild-type mice intraperitoneally administered with a dose of hApoA-I (Lee-Rueckert et al., 2011). The generation of genetically-modified mice for ABCA1, apoA-II, CETP, hepatic lipase (HL) and EL has permitted to identify potential molecular targets for modulating macrophage-specific RCT and HDL antioxidant activity (see Table 1 for a summary).

ATP-binding cassette transporter A1 (ABCA1)

ABCA1 is present in liver, intestine and others peripheral tissues, particularly in macrophages. ABCA1 is required

	HDL-C	HDL antioxidant potential	Macrophage-to-feces RCT
ABCA1 deficiency (Calpe-Berdiel et al., 2005)	↓	Non determined	↓
Human Apo A-II overexpression (Ribas et al., 2004; Rotllan et al., 2005)	↓	↓	=
CETP overexpression (Rotllan et al., 2008)	↓	=	=
HL and EL overexpression (Escola-Gil et al., 2013b)	↑	↑	↑

Table 1. Assays of HDL function in genetically-modified mice.

for the maintenance of plasma HDL levels via pre β -HDL formation (Figure 1), which are among the main acceptors of cholesterol efflux (Basso et al., 2003). Macrophage-selective inactivation of ABCA1 promotes atherosclerotic lesions without affecting plasma HDL levels (Aiello et al., 2002; Francone et al., 2005). We investigated the *in vivo* role of ABCA1 in macrophage-specific RCT by using the wild-type ABCA1^{+/+}, ABCA1^{+/-} and ABCA1-deficient (ABCA1^{-/-}) mice (Calpe-Berdiel et al., 2005). Whole body cholesterol balance did not differ among genotypes, indicating that total RCT does not seem to be affected by ABCA1 or HDL deficiency. However, a direct relationship was observed between ABCA1 gene dose and the amount of macrophage-derived [3H]-cholesterol in plasma. Importantly, ABCA1^{-/-} mice had a significantly reduced excretion of fecal [3H]-cholesterol (Calpe-Berdiel et al., 2005). In line with these findings, selective inactivation of ABCA1 in macrophages also impaired macrophage-to-feces RCT (Wang et al., 2007). Overall, these findings confirm the crucial role of ABCA1 in this atheroprotective mechanism of HDL (Lee-Rueckert et al., 2016).

Apolipoprotein A-II

In contrast with the cardioprotective effect of apoA-I, the overexpression of mouse and human apoA-II has usually been found to be proatherogenic (Warden et al., 1993; Escola-Gil et al., 1998). However, the role of apoA-II on major HDL functions remains unclear. One of the mechanisms by which apoA-II cause atherosclerosis may be an impaired ability of HDL to protect against low density lipoprotein (LDL) oxidation, as reported in mouse apoA-II transgenic mice (Castellani et al., 1997). We investigated the potential of human

apoA-II (hApoA-II) transgenic HDL to protect against oxidative modification of apoB-containing lipoproteins (Escola-Gil et al., 2000). We found a significant increase in the amount of aortic antigens related to LDL oxidation in transgenic mice overexpressing hApoA-II. HDL of transgenic mice failed to protect the apoB-containing lipoproteins from oxidation, including very low-density lipoprotein (VLDL) and LDL. Human apoA-II-containing HDL also showed a decreased content of the main HDL antioxidant enzymes paraoxonase/arylesterase 1 (PON1) and platelet-activating factor-acetylhydrolase (PAF-AH). Incubating isolated hApoA-II with control plasma at 37°C decreased PON1 activity in plasma and HDL. The displacement of PON1 by physiologic concentrations of hApoA-II could explain why PON1 is mostly found in HDL particles without apoA-II, as well as, the lack of antiatherogenic properties of apoA-II-enriched HDL (Ribas et al., 2004). In an independent study, we investigated the effects of hApoA-II on the entire RCT pathway by using transgenic mice under chow or an atherogenic diet. On the chow diet, hApoA-II overexpression accelerated the transfer of macrophage-derived cholesterol to liver and feces. However, the magnitude of macrophage-to-feces RCT did not differ between transgenic and control mice fed the atherogenic diet. Taken together, our results indicate that hApoA-II exerts its proatherogenic effect by counteracting antioxidant properties of HDL rather than by impairing macrophage-to-feces RCT (Escola-Gil et al., 2000; Ribas et al., 2004; Rotllan et al., 2005).

Cholesteryl ester transfer protein (CETP)

CETP promotes the removal of cholesteryl ester from HDL in heteroexchange for triglycerides derived from LDL,

VLDL or chylomicrons (Figure 1). CETP transgenic mice, which naturally do not have this transporter protein, showed increased susceptibility to atherosclerosis, although the effects of CETP on CVD in human studies remain unclear (Tall, 1993; de Grooth et al., 2004). We determined the influence of CETP activity on the two major antiatherogenic functions of HDL by using transgenic mice overexpressing CETP. The magnitude of macrophage-derived cholesterol in liver and feces did not differ between CETP transgenic mice and control mice and this was independent of the diet used (chow or atherogenic). Furthermore, the injection of endogenous CETP-expressing macrophages did not alter macrophage RCT in control mice. HDL from CETP-expressing mice protected LDL from oxidative modification at similar levels that did the HDL from control mice (Rotllan et al., 2008). Although we did not find significant evidence that CETP affected these major HDL functions, adenovirus-mediated human CETP expression promoted the macrophage-dependent RCT rate (Tanigawa et al., 2007; Tchoua et al., 2008). In any event, these results would not predict the increased atherosclerosis susceptibility of CETP transgenic mice.

Hepatic lipase (HL) and endothelial lipase (EL)

HL and EL are part of the triglyceride lipase family and differ in their hydrolytic activities. EL is mainly a phospho-

lipase enzyme while HL has phospholipase and triglyceride lipase activities; both enzymes may convert large HDL to smaller HDL (Yasuda et al., 2010). HL- and EL-deficiency in mice caused a significant increase on HDL lipid and apoA-I levels (Ishida et al., 2003). A physiologically important question is whether HL and EL activities alter HDL cardioprotective functions. We evaluated the effects of HL and EL deficiency on macrophage-to-feces RCT pathway in vivo, the susceptibility to oxidation of HDL, and its ability to protect against LDL oxidation (Escola-Gil et al., 2013b). As expected, both HL- and EL-deficiency caused an increase in cholesterol and phospholipids associated to HDL and an increase in HDL size. ApoA-I and PAF-AH were also increased in both HL- and EL-deficient mice but no changes in PON1 activity were found. These changes did not correlate with alterations in the liver expression of enzymes involved in HDL metabolism. These genetically-modified mice displayed increased levels of macrophage-derived HDL-bound [3H]-cholesterol which was concomitant with increased levels of [3H]-cholesterol in feces. HDL from the HL- and EL-deficient mice was less prone to oxidation and had a higher ability to protect LDL from oxidation. These changes were more pronounced in the EL-deficient mice (Escola-Gil et al., 2013b). However, the role of HL- and EL-deficiency on atherosclerosis development provided divergent data

	Drug	HDL-C	HDL antioxidant potential	Macrophage-to-feces RCT
Fibrates (Rotllan et al., 2011)	Fenofibrate	↑	Human apoAI, PLTP and liver ABCG5/G8 ↑	↑
	Gemfibrozil	=	Liver ABCG5/G8 ↑	=
LXR agonist (Calpe-Berdiel et al., 2008)	T0901317	↑	Liver ABCA1, ABCG5/G8 and ABCG1 ↑ and intestine ABCA1 and ABCG1 ↑	↑
SERMs (Fernandez-Suarez et al., 2016)	Tamoxifen	↓	Liver ABCG5/G8 ↓	↓
	Raloxifen	↓		=
Cholesterol absorption (Silvennoinen et al., 2012)	Ezetimibe	=	Intestinal NPL1C1 ↓	↑
	PPARδ agonist GW0742	=	Intestinal NPL1C1 ↓	↑

Table 2. Effects of therapeutic agents on macrophage-to-feces RCT.

since other HDL-independent biological properties of HL and EL, such as their role in modulating apoB-containing lipoprotein catabolism, macrophage cholesterol uptake and inflammation influence (Annema and Tietge, 2011).

Pharmacology

RCT-enhancing therapies are currently considered a promising strategy for the prevention and treatment of atherosclerotic CVD. An important number of RCT-targeted drugs have been used in mice to test their effects on macrophage-specific RCT in vivo. Some of these therapeutic approaches included fibrates, liver X receptor (LXR) activation and selective estrogen receptor modulators (SERMs), and cholesterol absorption inhibitors (Table 2).

Fibrates

Fibrates are peroxisome proliferator-activated receptors alpha (PPAR α) agonists commonly used mainly in hypertriglyceridemic patients. Clinical effects of fibrates are attributed to their ability to reduce triglycerides and increase HDL-C levels (Fruchart et al., 1999; Staels et al., 2008). Current guidelines recommend the use of fenofibrate for preventing cardiovascular disease, particularly for high-risk statin-treated patients with atherogenic dyslipidemia. Furthermore, fenofibrate appears to exert a stronger effect on plasma hApoA-I levels than other fibrates. However, evidence that fibrates reduce mortality and morbidity associated with CVD is still unclear (Grundey et al., 2004; Duez et al., 2005).

Since HDL metabolism is regulated in an opposite manner by fibrates in wild-type rodents due to sequence divergences in the mouse *Apoa1* promoter, our group investigated the effects of fibrates on the entire macrophage-dependent RCT pathway in mice overexpressing hApoA-I, a model that elicits a humanized response to fibrates (Rotllan et al., 2011). We used hApoA-I transgenic mice treated with gemfibrozil, fenofibrate or vehicle as control group. We found that fenofibrate increased significantly macrophage-derived plasma [3H]-cholesterol 24 and 48 hours after macrophage injection and the net fecal [3H]-cholesterol + bile acid excretion over 48h. Both fibrates increased mRNA levels of liver *Ppara* and two key liver genes involved in hepatic cholesterol transport to bile and feces, *Abcg5* and *Abcg8*; but only fenofibrate induced lipid profile modifications. After fenofibrate treatment, total plasma hApoA-I and HDL-C levels in female transgenic mice were higher than those of vehicle-treated mice. Fenofibrate also caused an increase in

the amount of nascent pre β -HDL particles and plasma phospholipid transfer protein (PLTP) activity, concomitant with an upregulation of *Pltp* and human *APOA1* gene expression. In contrast, gemfibrozil did not affect these plasma parameters and the macrophage-derived [3H]-cholesterol flux to plasma and feces of hApoA-I transgenic mice. Unlike gemfibrozil, fenofibrate also induced the generation of larger HDL particles, which were more enriched in cholesteryl esters, and promoted macrophage cholesterol efflux to plasma in vitro. None of the drugs affected net intestinal cholesterol absorption. These findings demonstrated that fenofibrate, but not gemfibrozil, promoted in vivo macrophage-specific RCT, thereby highlighting the differential action of this fibrate on HDL functionality. These data would be consistent with a protective clinical effect of fenofibrate that remains to be clearly demonstrated in interventional human studies (Rotllan et al., 2011).

Liver X activation

The LXR α and β are oxysterol-activated nuclear receptors that regulate the expression of a number of genes involved in RCT such as ABCA1, ABCG1, ABCG5, ABCG8 and cytochrome P450 7a1 (CYP7A1) (Repa et al., 2002). LXR α is expressed mainly in liver and secondarily in adrenal glands, intestine, adipose tissue, macrophages, lung and kidney. LXR β is expressed in almost all tissues (Zelcer and Tontonoz, 2006). Overexpression of ABCG5/G8 or activation of LXR α results in increased hepatobiliary excretion of cholesterol and increased fecal neutral sterol excretion (Yu et al., 2002a). Consistent with these findings, the inactivation of these two genes has opposite effects on sterol hepatobiliary excretion (Yu et al., 2002b).

Synthetic high-affinity LXR α β agonists promote RCT from macrophages to feces in vivo despite having a limited impact on HDL-C levels. These specific activators also inhibit atherosclerosis progression, and even promote atherosclerosis regression in mice (Terasaka et al., 2003). The results of these studies support the concept that LXR activation promotes macrophage-specific RCT pathway by increasing biliary cholesterol excretion through ABCG5/G8 upregulation (Yu et al., 2003).

We initially investigated the effects of ABCG5/G8 deficiency as well as those of LXR agonist induction of RCT from mouse macrophages to feces in ABCG5/G8-deficient (ABCG5/G8 $^{-/-}$) and wild-type (ABCG5/G8 $^{+/+}$) mice (Calpe-Berdiel et al., 2008). When [3H]-cholesterol-labeled mouse macrophages were injected intraperitoneally into the ABCG5/G8 $^{+/+}$ and ABCG5/G8 $^{-/-}$ mice, we found an in-

creased radiolabeled HDL-bound [3H]-cholesterol after the label injection. However, not significant differences were found between the two groups in terms of [3H]-tracer in liver, feces and bile acids. These findings were rather surprising, considering that disruption of ABCG5/G8 resulted in a marked reduction in biliary cholesterol levels. This could indicate an important role of the intestine in the model used in this study. Recent data suggest that the intestine may play an important role in excreting cholesterol from plasma to the intestinal lumen and feces. Then, we investigated whether ABCG5/G8 were necessary for LXR-mediated induction of macrophage-specific RCT pathway by injecting the radiolabeled macrophages into the ABCG5/G8^{+/+} and ABCG5/G8^{-/-} mice treated with or without the LXR agonist T0901317. Treatment with T0901317 increased liver ABCG5/G8 expression, and was associated with a 2-fold increase in the fecal excretion of macrophage-derived [3H]-cholesterol of ABCG5/G8^{+/+} mice. However, the LXR treatment did not affect fecal [3H]-cholesterol excretion in ABCG5/G8^{-/-} mice. We also determined the fate of [3H]-cholesterol from HDL by radiolabeling HDL with HDL-[3H]-cholesteryl oleate. Untreated and T0901317-treated ABCG5/G8^{+/+} and ABCG5/G8^{-/-} mice were injected intravenously with the radiolabeled HDL. Plasma clearance of intravenously injected [3H]-HDL was significantly slower in treated ABCG5/G8^{+/+} mice compared with untreated mice. However, LXR activation did markedly increase the recovery of HDL-derived [3H]-cholesterol in the feces of ABCG5/G8^{+/+} mice. Conversely, LXR agonists did not have the ability to increase fecal [3H]-cholesterol in ABCG5/G8^{-/-} mice (Calpe-Berdiel et al., 2008)

Our results demonstrated that ABCG5/G8 transporters are essential for the LXR agonist-mediated induction of RCT from macrophages to feces *in vivo*, thereby suggesting that upregulation of ABCG5/G8 may be an effective therapeutic strategy to increase macrophage-to-feces RCT pathway and reduce atherosclerosis. However, LXR activators have undesired effects such as hepatic triglyceride accumulation. Since LXR β activation may target intestine, promote macrophage RCT and prevent atherosclerosis, current efforts have focused on developing novel LXR β -selective ligands that circumvent liver side effects. In contrast, somewhat surprisingly, ABCG5/G8 deficiency in the mouse did not affect RCT from macrophages.

Selective estrogen receptor modulators (SERMs)

SERMs are non-steroidal molecules that can be estrogen-agonists or antagonists depending on the tissue targeted and the kind of estrogen receptor (ER). Some examples

of SERMs are tamoxifen (TAM) and toremifene (TOR), which are mainly used in breast cancer, and raloxifene (RAL), which is used for the treatment of osteoporosis in postmenopausal women (Pickar et al., 2010). Beyond their therapeutics effects, SERMs also reduce cholesterol associated to LDL-C. This effect may be mediated by the decrease of cholesterol biosynthesis, the increase of the expression of LDL receptor (LDLR), and the inhibition of cholesterol acyl transferase (ACAT) (Suarez et al., 2004; Cerrato et al., 2015).

We have investigated whether the SERM-mediated interference of cholesterol trafficking in macrophages may restrict cholesterol efflux to HDL and, consequently, impair the cholesterol transport to feces *in vivo*. As expected, SERMs impaired intracellular acetylated LDL (acLDL)-derived cholesterol trafficking and cholesterol efflux in macrophages (Fernandez-Suarez et al., 2016). Human THP-1 macrophages were loaded with [3H]-cholesterol-labeled acLDL in the presence of SERMs and cholesterol efflux to different acceptors was determined. TAM decreased apoA-I-mediated cholesterol export in a dose-dependent manner, and HDL-mediated cholesterol efflux was also reduced. RAL and TOR also decreased apoA-I-mediated cholesterol efflux, but not the HDL-mediated cholesterol efflux. acLDL markedly increased the protein levels of ABCA1 and ABCG1 in macrophages, whereas TAM, RAL and TOR had the opposite effect. The TAM-mediated effect on macrophage [3H]-cholesterol efflux was not related with its effects on cholesterol intracellular trafficking. Therefore, SERMs impaired cholesterol export from THP-1 macrophages by two ways: lowering the availability of lipoprotein cholesterol and impairing the acLDL-mediated induction of ABCA1 and ABCG1. Moreover, SERMs effects were ER independent because ESR1 and ESR2 expression was undetectable in THP-1 macrophages and the addition of an ER down-regulator did not alter the effect of any SERM. These effects were also confirmed in human monocyte-derived macrophages. Importantly, when [3H]-cholesterol-loaded macrophages were intraperitoneally injected into mice, TAM, but not RAL, decreased the [3H]-cholesterol levels in serum, liver and feces. This effect was primarily attributable to the TAM-mediated reduction of the capacity of HDL to promote cholesterol mobilization from macrophages (Fernandez-Suarez et al., 2016).

Cholesterol absorption inhibitors

Both macrophage- and non-macrophage-derived cholesterol molecules are transferred through multiple steps of the reverse pathway to the intestine. On average, 50% of the cholesterol present in the intestinal lumen may be reabsor-

bed, and the remainder will be ultimately excreted from the body as fecal cholesterol to complete the RCT pathway. The final regulatory steps of RCT that occur in the small intestine play an important role in determining the efficiency of cholesterol absorption and thus can influence the rate of the total body RCT (Lee-Rueckert et al., 2013). We have recently investigated the effects of modulating the traffic of cholesterol across the enterocyte on the rate of the specific itinerary of cholesterol from peripheral macrophages into feces. Therefore, interventions that inhibit cholesterol absorption including ezetimibe administration and PPAR β/δ activation with GW0742 increase the excretion of macrophage derived cholesterol in feces by reducing intestinal Niemann-Pick C1-like protein 1 (NPC1L1) activity (Silvennoinen et al., 2012). Furthermore, certain pathophysiological conditions associated with an increased risk of atherosclerotic CVD have been found to modify intestinal cholesterol absorption and affect the macrophage-to-feces RCT pathway (see Physiological stress section for details).

Physiological and physiopathological states

There are certain physiopathological states that may affect HDL functionality such as stress or the immunological and inflammatory processes. HDL function has been also related with the potential anti-tumorigenic role of HDL.

Physiological stress

In humans, a relationship between stress and atherosclerosis has been found in some epidemiological studies under conditions that trigger different types of stress (Rozanski et al., 1999). Simulated stress conditions in mouse models are useful for search mechanisms implicated in atherosclerosis. Previously studies indicate that the dual role of stress on atherosclerosis depends on the type of stress: it can be accelerated or remain unaffected (Kumari et al., 2003; Bernberg et al., 2009). We studied the rate macrophage-to-intestine RCT in mice exposed to restraint stress which mimics psychological stress in humans (Silvennoinen et al., 2012). We initially compared the results of a control mouse group without stress and mice exposed to physical restraint stress for 3 hours. Activation of hypothalamic-pituitary-adrenal (HPA) axis is the endocrine hallmark of stress and produce corticotrophin releasing factor (CRF) and corticosterone (CORT). In the stress group, CRF peaked and then rapidly returned to basal levels, whereas CORT levels remained elevated in stress group due to inhibition of

CRF by CORT. When [3H]-cholesterol-loaded macrophages were injected into the mice, stress did not affect cholesterol efflux from macrophages but inhibited intestinal cholesterol absorption thus leading to accumulation of intestinal [3H]-cholesterol in the stressed mice.

Furthermore, administration of CORT, the stress hormone in rodents, to non-stressed mice upregulated PPAR α and downregulated NPC1L1 protein levels in the small intestine, fully reproducing the effect of stress on the rate of macrophage-to-intestine RCT in the non-stressed mice. The acceleration of RCT induced by stress was fully inhibited in mice pretreated with a cytochrome P450 inhibitor, metyrapone. No additive increase of macrophage-to-intestine RCT rate occurred if the stress mice were treated with ezetimibe or the PPAR δ agonist GW0742 that blocks NPC1L1 action in the intestine (Silvennoinen et al., 2012). Overall, the results strongly indicated a functional connection between acute stress and decreased function of intestinal NPC1L1 via a mechanism likely mediated by PPAR α , which is known to negatively regulate NPC1L1 expression in mice. Determination the macrophage-to-intestine RCT rate in PPAR α knockout (KO) and LXR α KO mice exposed to stress shows that mere elevation of CORT by stress was not sufficient to promote macrophage-to-intestine RCT. Of note, we observed that the increase in the fecal excretion of macrophage-derived cholesterol induced by stress in mice was maintained for 7 days of chronic exposure (Silvennoinen et al., 2012).

Anti-tumorigenic role of HDL

Low levels of HDL-C have been associated with high risk of breast cancer (Ni et al., 2015). However the relationship between apoA-I and breast cancer remain unclear (Borgquist et al., 2016). ApoA-I has anti-inflammatory, antioxidant or anti-apoptotic properties. Some studies have demonstrated that hApoA-I-containing HDL has potent anti-tumor activity in xenograft mouse models of ovarian cancer and mouse models of malignant melanoma and Lewis lung carcinoma. ApoA-I mimetic peptides, which mimic the distribution of the charge and structure of portions of apoA-I, are considered as potential therapeutic agents for preventing a variety of inflammation-related diseases, including cancer. Indeed, two reports have demonstrated that the apoA-I mimetic peptide 4F significantly reduce tumor growth in xenograft mouse models of ovarian and colon cancer (Van Lenten et al., 2009; Su et al., 2010).

We have recently investigated the effect of apoA-I overexpression or administrated 4F in a validated mouse mammary tumor virus (MMTV) polyoma middle T antigen

(PyMT) transgenic mouse model that spontaneously develop adenocarcinomas in the mammary gland. The mimetic peptide 4F increased tumor latency, decreased the mammary gland weight and total tumor burden compared with vehicle treatment. However, hApoA-I did not produce any effect on tumor onset and growth despite increasing HDL-C. The anti-tumorigenic effects of 4F were closely associated with its ability to reduce the oxidized (ox) LDL (Cedo et al., 2016). Overexpression of hApoA-I also reduced the formation of oxLDL. However, the levels of 27-hydroxycholesterol (27-HC), a metabolite that has been reported to promote tumor growth in mouse models of ER-positive breast cancer (Nelson et al., 2013), were higher both in the serum and mammary tissue of PyMT-hApoA-I mice compared to control mice. The expression of *Cyp7b1*, the main enzyme implicated in 27-HC catabolism, was downregulated in mammary tissue of hApoA-I mice. This raised 27-HC could counteract the HDL-mediated benefits on the oxLDL levels and tumor progression and explain that the apoA-I mimetic peptide 4F, but not increased apoA-I-containing HDL levels, inhibits tumor growth in mice with inherited breast cancer. Our data are consistent with the reported anti-tumorigenic activity of apoA-I mimetics in other type of cancers (Cedo et al., 2016).

Immune response

Mast cell activation and vascular permeability.

Mast cells are a type of white blood cell that function as a “master regulator” of the immune system. They are present in connective tissues around the body and in the arterial intima; mast cells have been found closely to macrophage foam cells (Kaartinen et al., 1995). Mast cell activation lead to a degranulation and chymase contained in its cytoplasm was able to modify HDL particles in vitro and to inhibit macrophage cholesterol efflux (Lee-Rueckert and Kovanen, 2006). We investigated whether local activation of mast cells would attenuate cholesterol efflux from neighboring macrophage foam cells, thereby disrupting the entire macrophage-to-feces RCT pathway (Lee-Rueckert et al., 2011). First, we demonstrated that the injection of mast cell-degranulating compound 48/80 in the peritoneal cavity of mice was able to inactivate by proteolysis the activity of apoA-I as an ex vivo cholesterol acceptor. Then, we evaluated the effects of injecting 48/80 into the C57Bl/6J mice to induce peritoneal mast cell activation, hApoA-I to stimulate RCT, and [3H]-cholesterol labeled J774 macrophages for measurement of the rate of RCT. After 3 hours, [3H]-radioactivity was measured in the intestinal lumen.

The hApoA-I stimulus on RCT was completely blocked in 48/80-treated mice with competent mast cells. In mast cell-deficient mice this RCT stimulated by apoA-I is higher in 48/80-treated mice indicating that the impaired RCT rate in 48/80-treated wild type mice was mast cell-dependent. Pre-incubation hApoA-I with or without a mouse chymase and posterior injection to mouse confirmed that impaired RCT was a consequence of apoA-I proteolysis. Although the fragments of apoA-I in peritoneal fluid could not be identified due to their rapid clearance, the study allowed to validate the hypothesis that activation of mast cells causes apoA-I proteolysis, and as a result, there is a decrease in the RCT pathway (Lee-Rueckert et al., 2011).

The concentration of lipoprotein particles in the interstitial fluid is the result of a dynamic balance between entry via the capillaries and the exit via lymphatic system (Miller et al., 2011). Skin and intimal layer of arteries are two key points for cholesterol accumulation and may share common pathogenic mechanisms. HDL is removed from interstitial fluids by the lymphatic system and it has been shown that variation in its flow may stimulate RCT (Martel et al., 2013). We have recently investigated whether an increased vascular permeability promotes RCT from skin. Therefore, we evaluated the effect of increased vascular permeability in the skin on the RCT rate from J774 foam cells injected into the dorsal subcutaneous layer of the skin (Kareinen et al., 2015). The increase of the subcutaneous levels of histamine promoted HDL influx to skin and accelerated the rate of RCT from skin to feces. The treatment with different histamine-receptor antagonists indicate that the stimulatory effect of histamine on RCT was mainly mediated by histamine H1 receptor. These results were also found when other vasodilator agents were injected, such as serotonin and bradykinin. Exogenous administration of HDL and histamine in apoA-I deficient mice evidenced that the increase of circulating HDL levels and triggering of local vascular permeability were both required to enhance macrophage RCT from subcutaneously located macrophages. Importantly, the degranulation of activated mast cells releases a number of vasoactive components, including histamine, which was able to stimulate endogenously the RCT pathway (Kareinen et al., 2015). This contrasts with our previous findings that demonstrated the mast cell activation cause impaired macrophage-to-intestine RCT when hApoA-I was intraperitoneally injected to stimulate RCT (Lee-Rueckert et al., 2011). The role of mast cell activation may probably depend on the balance between vascular permeability and apoA-I proteolysis.

Autoimmune arthritis

The relationship between dyslipidemia and the incidence of CVD in patients affected by rheumatoid arthritis has been established but it is still the subject of intense debate (John and Kitas, 2012). ApoE and inflammatory immune response are inversely correlated; however, the mechanism involved in this process remains unclear (Postigo et al., 2011). We recently investigated the severity of one type of autoimmune arthritis, Collagen-II-autoimmune arthritis (CIA), in apoE-deficient mice. ApoE deficiency was associated with higher severity of CIA (Alvarez et al., 2016). Consistent with these findings, a significantly increase in the expression of *arthritogenic Il1 β , Tnfa and Il6* was observed in apoE-deficient mice, thereby indicating that partial or total apoE deficiency modifies the polarization of macrophages after a potent inflammatory insult. ApoE-deficiency also altered the biochemical composition of HDL particles. However, the lipoprotein particles showed similar antioxidant ability, suggesting that the exacerbation of arthritis is largely independent of HDL function. We analyzed the role of hypercholesterolemia in the severity of CIA by using LDLR-deficient mice. The fact that CIA was more severe in apoE-deficient mice than in LDLR-deficient mice excludes that the LDLR is the mechanism by which ApoE modulates CIA severity. These findings demonstrate that both hypercholesterolemia and ApoE, but not HDL function, regulate the intensity of systemic autoimmune response (Alvarez et al., 2016).

Diabetes

Patients with type 2 diabetes (T2D) show an increased risk for premature CVD and death (Mazzone et al., 2008). Most (~80%) of individuals with T2D are obese, highlighting the pivotal role of increased adiposity as a risk factor (Chadt et al., 2000). Plasma levels of lipids are also frequently altered in both entities (Bays et al., 2013; Tchernof and Despres, 2013), particularly, those of HDL which are often reduced (Kontush and Chapman, 2008). HDL are also dysfunctional in patients with T2D (Kontush and Chapman, 2008). Consistently, cholesterol efflux mediated by HDL from diabetic patients is markedly reduced (Apro et al., 2016). In relation to T2D, compelling experimental evidence suggests that the hepatobiliary trafficking of cholesterol might be impaired in an animal model, the db/db mice, being in part associated with reduced hepatic levels of ABCG5 and G8. We have recently demonstrated that macrophage-to-feces RCT is impaired in db/db mice partly due to defects in the hepatic LXR signalling (Errico et al., 2017). In this case, RCT dynamics may be partly restored by a potent LXR agonist who induces *Abcg5/g8*. No-

tably, a commensurate favourable up-regulation in the hepatic gene expression of these two transporters, and other known targets of LXRA, was also observed in obese patients subjected to bariatric surgery along with an improvement in their fatty liver phenotype. Additionally, reduced liver *Abcg5/g8* protein abundance in db/db mice has been also partly explained by the participation of post-transcriptional mechanisms involving enhanced endoplasmic stress (Sabeva et al., 2009). Interestingly, the induction of LXR has been also reported to ameliorate ER stress induced by obesity and hepatic lipid accumulation in vivo (Rong et al., 2013); potentially, this might help in the partial restoration of liver ABCG5/G8 protein abundance in LXR-treated db/db mice. Taking together, these data might support the potential of targeting LXR signalling as a strategy to improve RCT in diabetes.

Dietary factors

Significant evidence has permitted to identify associations between serum HDL-C and diet, revealing a link between different dietary factors and their potential effects on HDL-mediated atheroprotection (Escola-Gil et al., 2015)

Dietary fats

The content of the dietary fat plays a key role in the development of CVD and metabolic diseases, such as obesity, atherosclerosis and T2D. Dietary saturated fatty acids (SFA) intake is associated with an increased risk of CVD. Also, a direct relationship between high intake of SFA and HDL-C has been established from several epidemiological studies and one meta-analysis (Knuiman et al., 1987; Mensink et al., 2003; Kotseva et al., 2008). Conversely, polyunsaturated fatty acid (PUFA) intake usually reduces HDL-C, even though they are potentially atheroprotective (Escola-Gil et al., 2015).

We initially investigated the effect of a high-SFA, Western-type diet, with or without added cholesterol, on the entire macrophage-to-feces RCT pathway in mice (Escola-Gil et al., 2011). The high-SFA and cholesterol-containing diet caused a significant increase in plasma cholesterol, HDL-C, and liver cholesterol and accelerated the rate of macrophage-to-feces RCT by 3- to 4-fold. These effects were greatly reduced in mice fed the same high-SFA diet without added cholesterol. We also used humanized mice expressing human CETP and, similarly to the findings in wild-type mice, the high-SFA and cholesterol-containing diet induced a significant increase in fecal macrophage-derived [3H]-tracer excretion. The dietary stimulus on RCT was also almost totally absent in CETP transgenic mice fed with the

SFA diet without cholesterol. When we analyzed the different RCT steps, the serum from mice fed the high-SFA and cholesterol-containing diet had a higher ability to induce macrophage cholesterol efflux. Furthermore, the high-SFA and cholesterol-containing diet-fed mice showed an accelerated excretion of fecal HDL-derived [3H]-cholesterol. The latter was completely blunted when mice were fed the diet without cholesterol. Importantly, only the high-SFA and cholesterol-containing diet increased liver *Abcg5/g8* expression. We also found that this RCT increase was independent of other existing metabolic disarrangements such as obesity or insulin resistant. We concluded that the presence of dietary cholesterol and liver ABCG5/G8 transporters is required for the SFA- and cholesterol-mediated induction of RCT. Since this diet also promoted atherosclerosis, this change in RCT seems to constitute a compensatory mechanism to protect macrophages from cholesterol accumulation (Escola-Gil et al., 2011).

Since the high-SFA and cholesterol-containing diet impairs HDL antioxidant function, we investigated whether PUFA could correct the deleterious effects of SFA. We evaluated the effect of replacing dietary SFA by PUFA (mainly the linoleic and α -linolenic acids) on HDL antioxidant potential and the macrophage-specific RCT pathway (Cedo et al., 2015). The high-SFA diet caused a significant increase in serum HDL lipids and upregulated the levels of oxidized (ox) HDL and oxLDL. Replacing dietary SFA with PUFA reverse the effects of SFA on HDL lipid levels and on lipoprotein oxidation. The ability of HDL to reduce LDL oxidation was evaluated by conjugated diene formation of LDL under prooxidant conditions in the presence of HDL. The results demonstrated that HDL from high-SFA and cholesterol-containing diet-fed mice showed an impaired ability to protect against LDL oxidation. Importantly, the PUFA-containing diet prevented the SFA-mediated impairment of HDL antioxidant function. The preferential action of PAF-AH on linoleic acid-containing phospholipids of oxidized lipoproteins might explain the higher ability of PUFA-fed mice HDL to prevent the onset of LDL oxidation. These PUFA-mediated changes in oxidized lipoproteins were not associated with the insulin resistance. In contrast with the critical role of cholesterol on macrophage-to-feces RCT, the amount of dietary PUFA did not affect the rate of RCT pathway in mice (Cedo et al., 2015).

Phytosterols

Dietary phytosterols are able to reduce intestinal cholesterol absorption and consequently decrease the LDL levels on serum (Calpe-Berdiel et al., 2009). Beyond this potential

cardioprotective role in CVD, some epidemiological studies have suggested that phytosterols may have anti-tumorigenic properties (Berges et al., 1995). Some potential mechanisms include alteration in sterol metabolism which may affect cancer cell growth, apoptosis promotion and decreased angiogenesis (Bradford and Awad, 2010). We analyzed the role of phytosterol-supplemented diet in the tumor onset and progression in PyMT mice (Llaverias et al., 2013). A 2% phytosterols supplement caused a significant delay in the development of hyperplastic mammary lesions and a decrease in total tumor burden in PYMT mice fed a high-fat, high-cholesterol diet. Also, Cyclin D1, a tumor biomarker, was shown to be significantly decreased in PyMT transgenic mice fed the phytosterol supplement. However, phytosterols neither affected the availability of cholesterol in the mammary gland nor altered the expression of the main cholesterol transporters. Importantly, phytosterols reduced HDL-peroxidation, thereby enhancing HDL antioxidant capacity. Decreased values of Laudan GP indicated that this protection might be due to changes in the physicochemical properties of HDL, thereby increasing the packing of the surface lipids and promoting lipoprotein resistance to oxidation. We concluded that dietary phytosterols caused a delay on breast tumor progression in the setting of a typical Western diet and suggest that phytosterols may exert these anticancer effects by preventing oxidative damage. (Llaverias et al., 2013)

Methionine

Methionine-rich foods can affect the plasma levels of homocysteine (Hcy), an intermediate metabolite of the folic acid pathway, causing a hyperhomocysteinemia (HHcy). HHcy induced by methionine can accelerate atherosclerotic progression and consequently has been considered a risk factor for CVD (Dayal and Lentz, 2008). Furthermore, HHcy has been associated to low levels of HDL-C in animal models and in humans (Liao et al., 2007; Vergeer et al., 2010). We determined the effect of HHcy induced by a methionine-rich diet on macrophage-to-feces RCT and on the ability of HDL to protect against LDL oxidation. Methionine-induced HHcy in mice resulted in a significant decrease of HDL-C and apoA-I. The susceptibility of HDL to oxidation was enhanced in HHcy mice. This change was concomitant with an impaired ability of HDL to prevent LDL oxidative modification. Importantly, PON1 and PAF-AH activities, two of the main HDL-associated enzymes with antioxidant activity, were also reduced in HHcy mice. The ability of HDL to efflux cholesterol from macrophages was decreased in HHcy mice; however, dietary methionine did

not affect the macrophage-specific RCT measured as the output of macrophage-derived cholesterol into feces (Julve et al., 2013). Overall, our findings indicate that HHcy might exert at least part of its proatherogenic effect by impairing the antioxidant properties of HDL.

Resveratrol

Resveratrol, a natural polyphenolic compound, is found in foods such as the skin of grapes, blueberries, raspberries and mulberries. Some cardioprotective properties have been attributed to resveratrol, such as hypolipidemic, antioxidant and anti-inflammatory actions. Some studies in mice indicated that resveratrol may increase HDL-C and, consequently, inhibit atherosclerosis progression (Ramprasath and Jones, 2010). Resveratrol has also been identified as an activator of the NAD⁺-dependent deacetylase sirtuin1 (SIRT1), although resveratrol has not been proved to activate SIRT 1 directly. Therefore, both mechanisms may involve the modulation of LXR (Pacholec et al., 2010). We investigated the ability of resveratrol and SIRT1 expression to induce LXR-target genes and the macrophage-specific RCT pathway in vivo (Escola-Gil et al., 2013a). In contrast to the effects of the selective LXR-agonist T0901317, no changes in liver cholesterol levels and intestinal cholesterol absorption were observed in mice given resveratrol at different doses. The highest resveratrol dose did not increase HDL-C while the LXR agonist did cause a significant increase. Further, SIRT1 expression in transgenic mice did not affect serum HDL-C and intestinal cholesterol absorption compared with wild-type. However, SIRT1 transgenic mice showed higher non-HDL and liver cholesterol content which has been attributed to elevated liver lipogenesis (Qiang et al., 2011). As expected, mice treated with LXR-agonist presented a higher content of macrophage-derived [3H]-cholesterol in plasma and feces. However, macrophage-to-feces RCT rate was not affected by the treatment of resveratrol. Even though SIRT1 transgenic mice showed differences in the lipoprotein profile, SIRT1 expression did not affect the magnitude of macrophage RCT. Furthermore, the expression of LXR-target genes was unaffected by resveratrol. We concluded that resveratrol and SIRT1 expression did not promote the RCT pathway in vivo (Escola-Gil et al., 2013a).

Concluding remarks

Genetically engineered mouse models have been used extensively for investigating the role of different thera-

peutic approaches and the overexpression and deletion of HDL proteins involved in the major HDL cardioprotective functions. Current data have demonstrated that enhanced macrophage-to-feces RCT or HDL antioxidant functions are inversely correlated with atherosclerosis development. It should be noted that CETP expression did not affect major antiatherogenic functions in mice (Rotllan et al., 2008), which is consistent with the results of 3 synthetic CETP inhibitors that failed to reduce CVD (Barter and Rye, 2012; Lee-Rueckert et al., 2016). However, a new CETP inhibitor, anacetrapib, substantially increased HDL-C and apoA-I, but also pre- β HDL particles and reduced CVD events (Bowman et al., 2017). At present, other HDL-based therapies, such as infusions of apoA-I mimetics or other reconstituted HDL particles for increasing apoA-I levels, appear to be promising tools in preclinical studies. Taken together, the available data indicate that increased HDL-C do not always correlate with enhanced HDL functions and, therefore, should not be considered a biomarker of HDL functionality. In humans, the efficiency of RCT has been evaluated with the surrogate parameter that indicates the ability of HDL to promote the first step of RCT, the macrophage cholesterol efflux capacity. Recent clinical data suggest that this parameter is a strong predictor of CVD in humans (Rohatgi et al., 2014; Saleheen et al., 2015).

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